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TERATOGENESIS ASSAY- XENOPUS (FETAX)

PRINCIPAL INVESTIGATOR: John A. Bantle, Ph.D.

CONTRACTING ORGANIZATION: Oklahoma State University
Stillwater, OK 74078

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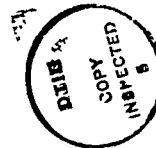
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| 19. ABSTRACT (Continue on reverse if necessary and identify by block number) The goal of the project was to develop and validate the Frog Embryo Teratogenesis Assay- <u>Xenopus</u> (FETAX). FETAX is a 96 h whole embryo bioassay designed to rapidly assess the developmental toxicity of pure compounds and complex mixtures. The five specific objectives of this study are: 1) validate FETAX using direct-acting compounds of known mammalian developmental toxicity, 2) develop and validate an <u>in vitro</u> metabolic activation system using rat liver microsomes, 3) explore and use three carrier solvents which will help solubilize non-polar compounds, 4) develop an "Atlas of Abnormalities" as a companion manual to the ASTM New Standard Guide for the Conduct of FETAX and, 5) compare the sensitivity of FETAX with a fathead minnow bioassay modified to have the same exposure conditions as FETAX. Eighteen test compounds were selected for FETAX validation. Each compound was tested using 1-2 range-finding tests with three definitive (over) | | | |
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tests. Endpoints of FETAX are mortality, malformation and growth inhibition. Compounds selected for testing included mammalian negatives and two strong positives. FETAX correctly identified the developmental toxicity of all compounds tested in the validation phase. One compound did require metabolic activation in order for it to test correctly.

The development of the in vitro metabolic activation system using Aroclor 1254-induced rat liver microsomes was completed and five compounds were successfully tested using this system. Additional work was done using isoniazid-induced rat liver microsomes which induced a cytochrome P-450j species not induced by Aroclor 1254. It was found that a 1:1 mixture of Aroclor 1254 and isoniazid-induced microsomes proved to be best activation system to bioactivate a broad range of chemicals. Some experiments were performed using phenobarbital and beta-naphthoflavone-induced microsomes as a substitute for Aroclor 1254 which suffers from waste disposal problems.

Triethylene glycol, dimethyl sulfoxide and acetone were evaluated as possible carrier solvents. Triethylene glycol proved to be the least toxic solvent but dimethyl sulfoxide had the best combination of low toxicity and solvation power. These solvents were tested in the presence of four known teratogens (trans-retinoic acid, methyl mercury chloride, trichloroethylene and 6-aminonicotinamide). Interactions ranging from synergism to antagonism were recorded for all combinations of solvent and teratogen. Only growth was not affected in this study. Mortality was significantly affected. It is recommend that when the use of a carrier solvent is required that two different solvents be used and the results compared.

The "Atlas of Abnormalities" was conceived as a companion manual to the ASTM New Standard Guide for the Conduct of FETAX. The guide helped to standardize the FETAX protocol and the manual provided valuable assistance in the area of animal husbandry and the recognition of malformations. The ASTM guide did not allow the publication of micrographs so the Atlas was a way of providing this information. Besides the collection of useful data, the Atlas has helped popularize the use of FETAX and has facilitated technology transfer.

The sensitivity of FETAX was compared with a fathead minnow bioassay modified to have the same exposure conditions as FETAX. Some question as to the sensitivity of FETAX was posed by Dr. Wes Birge of the University of Kentucky. However, different exposure times and incubation temperatures were used in his study which made interpretation difficult. In order to standardize these parameters in our study, we chose a five day exposure period (1 day longer than FETAX) and a constant 23-24°C incubation temperature. In two of three tests with different chemicals, FETAX was the most sensitive indicator of developmental toxicity.

As a result of this study, we conclude that FETAX should be immediately useful as an indicator of human health hazards (birth defects, low birth weight etc.) and as useful bioassay for use in ecotoxicology.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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INTRODUCTION

Project Goal and Objectives

The goal of this research project was to develop and validate an in vitro bioassay for developmental toxicants using embryos of the South African clawed frog Xenopus laevis. The assay will allow rapid screening of pure compounds or complex mixtures as part of the hazard assessment process. The assay has been named the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) by its developer Dr. James Dumont (1) of Oak Ridge National Laboratories.

Four objectives have been established for the present project. These are:

1. Validation: FETAX will be tested using a number of pure test chemicals whose mammalian developmental toxicity has been previously established. The rationale for this work is to establish the predictive accuracy of FETAX and establish the false positive and negative rate. Chemicals that do not test as predicted will be retested using an in vitro metabolic activation system used in conjunction with FETAX. In this manner the validation work will help in the development of the assay. Attainment of this objective will also help in future covalidation studies that will show that FETAX is reproducible and reliable. Abnormal embryos generated during this phase of the testing were photographed and the pictures used in preparing an "Atlas of Abnormalities" (objective #4).

2. Solvent Interaction Study: Some difficulty has been encountered in previous studies when non-polar test substances have been used. The use of dimethyl sulfoxide, acetone and triethylene glycol as carrier solvents was evaluated in this study. The no observable effects concentrations of each of these solvents has been determined but it was possible that these solvents acted synergistically and antagonistically with the test chemicals. It was an objective of this study to determine whether or not these type of interactions are occurring and how best to minimize them.

3. Validation of the Rat Liver Metabolic Activation System for FETAX: Aroclor 1254 induced rat liver microsomes are currently being used as an in vitro metabolic activation system for FETAX. Many proteratogens must be bioactivated before causing developmental effects while other compounds, such as nicotine, are rendered far less toxic. Since Xenopus embryos lack a functional metabolic activation system through the first four days of development, a substitute system must be exogenously provided. This system has been developed and used successfully. The objective in the present study was to validate the system in order to demonstrate that it increases the predictive accuracy of FETAX.

4. Atlas of Abnormalities: If FETAX is to provide repeatable and reliable data, it is imperative that a standardized methodology be provided to users. This guide is currently being developed through the auspices of the American Society for Testing and Materials. This new standard guide (See: Methodology Appendix) is nearly through the development cycle. During its development it became clear that new users were having difficulty judging which embryos were

malformed and which stages of development they were dealing with. Photographs taken for the present project have been published in a book which will be provided to interested parties for the purpose training FETAX technicians. The repeatability and reliability of FETAX are very dependent on the quality of the technical help. Because of the interrelationships between the Atlas and the Guide, much effort was spent on the development of the guide although it was not an original stated objective.

5. Comparative Sensitivity of FETAX: Midway through the contract, data emerged from Wes Birge's lab of the University of Kentucky suggesting that FETAX might be too tolerant as a developmental toxicity screen. However, Birge's group used different exposure lengths and other differing environmental conditions in carrying out their experiments. We designed follow on experiments comparing FETAX to fathead minnow development. We modified FETAX by adding an extra day to the exposure period and we modified the minnow early life stage test by reducing the exposure period by one day. In two of three cases tested, FETAX proved to be more sensitive and it was easier to conduct FETAX as well.

BACKGROUND

The Need to Screen for Teratogens

Approximately 70,000-110,000 chemicals are currently available in the marketplace with some 800 new chemicals released each year. Prior to their release into the environment, the safety of these chemicals must be firmly established. Because of the large numbers of chemicals to be tested and the even larger number of interactions possible when these chemicals are present in complex mixtures, in vivo assays employing mammals are not practical. The need for routine teratogenicity testing has led to the development of a number of in vitro teratogenesis assays that may prove useful in prioritizing compounds for further testing (1-7). Several years ago, Dumont and co-workers (1) developed and used the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) and applied it to screening complex environmental mixtures as well as pure compounds. We propose to further develop FETAX and evaluate this assay using compounds of known mammalian developmental toxicity. Successful development and validation of this assay will make available to the scientific community a four-day screening test providing reliable developmental toxicity data.

Development of FETAX as a Teratogenesis Screening Assay

In its present form, FETAX meets most of the criteria set forth by Kimmel et al. (8) for the validation of in vitro teratogenesis assays (8, 9-11). Endpoints such as mortality, malformation, growth, development and motor impairment are easily quantifiable and capable of exhibiting a dose-response relationship with the establishment of narrow confidence limits. Since many of the stages of amphibian development are similar to mammalian development, the "developmental relevance" of FETAX is higher (8) than many of the other teratogenesis assays. At present FETAX has a metabolic activation system using uninduced rat liver microsomes, but improvements can be made. Additional validation using compounds of known mammalian developmental toxicity needs to be performed. The successful

accomplishment of the specific aims listed below should make FETAX a fully developed and reliable teratogenesis screening assay.

The original work on developing an assay with Xenopus to detect environmental teratogens was performed in the laboratories of Greenhouse (7) and Dumont (12). Greenhouse used 48-hr exposures to military compounds (N-phenyl-a-naphthylamine and various hydrazines) to demonstrate toxic and teratogenic effects on developing embryos.

The early studies demonstrated that the Xenopus system can be used with a variety of chemicals and complex mixtures. The endpoints include: LC50 (mortality), EC50 (malformation-teratogenesis), no observable effects concentration (NOEC), growth (both length and developmental stage obtained in a given time period), motor behavior, pigmentation, and gross anatomy. The test chemical exposures are generally continuous for 96-hr (FETAX). Mortality and stage of development are checked at hours 24, 48, 72, and 96 hr, while the other endpoints are recorded only at 96 hr. Every 24 hr, fresh test compound and water is added (renewal). Data collection is simple as all observations are made with a dissection microscope. The data collected using FETAX are in harmony with the criteria for an in vitro teratogenesis screen given by Kimmel et al. (8). These include: good dose-response, adequate number of embryos, and easily defined endpoints.

FETAX Test Performance and Developmental Relevance

Dumont (unpublished) has validation data on 45 compounds with an approximate 85 percent correspondence to mammalian results. Sabourin (13-14) has completed testing of 32 compounds with 83 percent predictive accuracy. In our laboratory to date, we have tested 67 compounds and found that 60 gave results in FETAX consistent with the mammalian literature for a predictive accuracy rating of 89.5%. In at least two cases (isoniazid and pseudoephedrine), we feel that metabolic activation is involved and that the mammalian literature has not properly taken this into account. Resolution of these problems in validation would increase the predictive accuracy significantly. However, we are bounded by the limits of the accuracy of the mammalian literature since funds to repeat mammalian studies are lacking.

Correlation between laboratories has been encouraging. For example, Courchesne and Bantle (9) found a teratogenic index for hydroxyurea of 4.3, whereas Sabourin recorded 4.5 for the same chemical. In some cases, however, a lack of correspondence is apparent. Even though 5-fluorouracil tested strongly positive for teratogenesis in the laboratories of Bantle and Sabourin, the teratogenic indexes differed significantly. The reason for such discrepancies needs to be understood and the interlaboratory evaluation phase of the next contract will permit an evaluation of the inter-laboratory variability. If the assay is to be used for purposes beyond screening, such as for ranking teratogenesis of test chemicals, then the importance of minimizing variability in quantitative measurements emerges.

Additional evidence which supports the use of FETAX as a viable teratogen screen concerns its performance compared with that of other potential test systems and its developmental relevance. Sabourin et al. (10) found the Xenopus test emerged as the assay of choice when compared to the planaria system (3) and the Hydra system (15). These results can be attributed to

the numerous endpoints of the frog assay as well as its higher phylogenetic position. We have also found that endpoints in the Xenopus assay may demonstrate developmental relevance to those in mammalian systems. Sabourin and Carlton (unpublished) determined that the same stock of diphenylhydantoin caused pericardial edema as the primary endpoint in both the cultured whole rat embryo (16) and Xenopus embryos. We have also made this same observation. Dumont et al. (personal communication) similarly found that meclizine induced hydrocephalia in both frogs and mammals and that other teratogens produced similar abnormalities in both frogs and mammals. Sabourin (in preparation) has recently found that the total mammalian malformations (e.g., skeletal, visceral, nervous, etc.) caused by 17 teratogens were matched in Xenopus in 24/37 (65%) of the cases. Courchesne and Bantle (9) reported that a number of genotoxic chemicals caused the same general types of malformations in both Xenopus and rodent embryos. Lastly, Dawson et al. (17) have developed a artificial medium (FETAX solution) and have carried out a preliminary validation using five compounds ranging from a nonteratogen to a strong teratogen.

In Vivo Bioactivation

A principal objective of this proposed study is to develop a means of dealing with proteratogenic substances in the FETAX system. Preliminary work, done independently in the laboratories of Dumont and Sabourin, indicated that a limited degree of basal P-450 activity and subsequently induced P-450 activity is present in the 96-hr embryo. This activity is not great enough to qualify FETAX as a metabolically competent system however. A significant increase in mortality or malformation between 72 and 96 hrs does indicate that bioactivation may be occurring later in development. We have observed this increase in mortality between 72 and 96 hrs using histamine. Wesolowski and Lyerla (18) have also reported that hexokinase and alcohol dehydrogenase activities do not appear until the fifth day of development in Xenopus.

In Vitro Bioactivation

We have developed an in vitro metabolic activation system (MAS) for FETAX by using Aroclor 1254 induced rat liver microsomes cocultured with embryos (19). Initial attempts to use commercially available Aroclor 1254 induced rat liver S-9 supernatant (Litton Bionetics) failed because of high S-9 toxicity. We then attempted to further purify microsomes from uninduced rat liver homogenate in order to reduce toxicity (20,21). We were successful in this approach and able to activate the proteratogen, cyclophosphamide into its embryotoxic and teratogenic form as measured by FETAX (19,20). We used cyclophosphamide in the development of uninduced rat liver microsome system for FETAX. Cyclophosphamide is one of the consensus compounds listed by Smith et al. (22) for use in validating in vitro teratogenesis assays. That cyclophosphamide requires metabolic activation for its teratogenicity has recently been shown using rat embryos cultured in vitro (23,24). We duplicated these experiments with FETAX to facilitate the development of our metabolic activation system. A wealth of literature exists on not only the metabolism of cyclophosphamide but also its mutagenic and teratogenic potential (See: Mirkes (25) for review). This makes it much easier to interpret results. Cyclophosphamide is easily soluble in water, eliminating

the need for a carrier. Cyclophosphamide has now been adopted in our lab as a positive control to prove that each microsomal preparation is active.

Rat liver microsomes were prepared essentially by the method of Kitchen and Woods (20) and the method of preparation is presented in the Appendix (Section #1). Our chief contribution to the preparation and use of these microsomes in FETAX was the use of bovine serum albumin in reducing residual Aroclor to a level that does not affect experimental results and in the standardization of the amount of microsomal activity so that experiments could be repeated reliably (19). We have also established that microsomal activity lasts for five hrs at 24°C.

After developing the in vitro MAS for FETAX, a validation program was initiated to prove that the system yield the expected results. Proteratogens, such as cyclophosphamide, should increase in developmental toxicity upon bioactivation while others should be reduced. FETAX results using direct-acting developmental toxicants should not change upon addition of the in vitro MAS. Besides cyclophosphamide, we have also tested the proteratogens 2-acetylaminofluorene, rifampicin and benzo(a)pyrene in FETAX and obtained clear evidence of bioactivation for each (26). Both nicotine and cytochalasin D are examples of compounds that are inactivated by an in vitro MAS and we obtained similar results in FETAX (26,27). Lastly, we have already used ZnSO₄ as an example of a compound that should be unaffected by the MAS and obtained the expected results in FETAX (26). With Zn we had questioned whether or not rat liver metallothioneins would bind to the zinc and reduce its toxicity. This did not turn out to be a problem. Lastly, we have tested dilantin as an example of a compound that has both direct-acting and possibly developmentally toxic intermediates (28). Using specific inhibitors of P-450 enzymes, we were able to show that dilantin caused developmental toxicity in its parent form and that there was evidence for toxic intermediate but that the primary metabolites were not as toxic as the parent compound.

We did try to use Xenopus hepatocytes in a co-culture with Xenopus embryos as an alternate in vitro MAS. An advantage of using cultured hepatocytes as a bioactivating system would be the continuous production of the active metabolite as opposed to the use of rat liver microsomes where activation is limited to approximately 5 hrs (although fresh additions are made daily). However, Xenopus hepatocytes do not have as much inducible cytochrome P-450 as do rat liver microsomes and rat hepatocytes cannot be used because of the tonicity differences between amphibian and mammalian cells. Cell culture is far more expensive and technically demanding than the use of rat liver microsomes. Further, there are problems in plating an exact number of hepatocytes so that the generation of teratogen from proteratogen is repeatable. It must be remembered that every in vitro metabolic activation system has advantages and disadvantages and that no universally accepted system now exists that duplicates human metabolism.

At present, we feel that the use of Aroclor 1254 induced rat liver microsomes as an in vitro metabolic activation system for FETAX offers great promise. Additional validation compounds must be tested to ensure that there are no surprises that may affect testing results. However, assuming that there are none we feel that the MAS will greatly enhance the predictive accuracy of FETAX and extend the utility of the assay.

Military Significance

As stated in the USAMRDC Broad Agency Announcement, the proposed bioassay would fall under the area of Section C. Army systems hazards. This would include both health hazards of military material (part 2) and military environmental quality (part 4). Virtually any compound or mixture that is or can be made water soluble can be tested by FETAX for developmental effects.

FETAX is an alternative which offers some short and long-term solutions to current problems associated with developmental hazard evaluation. The problems are: increasing regulatory requirements, test cost, test duration, and the reduction in the use of mammals in research. Existing in vivo mammalian test systems are effective for the testing of drugs and cosmetics but are too lengthy for the screening of water samples and other complex mixtures. In addition, the number of chemicals in production which require testing is increasing at a faster rate than is feasible to handle with in vivo test systems. Animal rights groups have increased pressure to slow down or halt the use of mammalian in vivo test systems. These events have produced a climate which favors the use of alternative test systems for eventual refinement, reduction, and replacement of in vivo assays. In a study conducted for NIH, the National Academy of Sciences recently concluded that the development and use of lower form models for toxicity testing should be emphasized (29). Tighter restrictions will limit the use of in vivo assays and increase costs associated with their performance. The Office of Technology Assessment for the United States Congress has recently completed an assessment of the use of animals in testing, research, and education (30). Congressional options emanating from this study include support for the further development and use of in vitro test systems when appropriate.

Cost-effective, yet developmentally relevant, tests are urgently needed to allow the detection of developmental toxicants in the environment and to assess the developmental toxicity of pure compounds and drugs. FETAX offers the advantage of detecting the toxicity and teratogenicity of complex environmental mixtures where the individual action of each component may be known but the combined action cannot be predicted.

Because of its position on the phylogenetic scale, Xenopus provides the highest degree of developmentally relevant endpoints compared to other in vitro teratogenesis assays such as cell culture, planarian, fruitfly, and Hydra systems. The costs of performing the above mentioned tests are similar, except for cell culture which is higher. Xenopus can be considered a high connectivity model due to the amount of biological information available on this species (29). There is a good chance that observations made on this species can be connected to data from other organisms, including man. Xenopus has been a standard lab animal for numerous developmental studies and the Xenopus oocyte is a commonly used biomedical model. Consequently, much is known about the frog's morphology, physiology, and biochemistry. This cannot be stated for many of the alternative systems. Xenopus can be bred throughout the year and provides numerous offspring in order to facilitate statistical analysis. Because development is external, the speed of data collection is enhanced. The lack

of a placental relationship is a disadvantage, but this problem is shared with other emerging in vitro teratogenesis assays.

The research plan we followed was designed to improve and evaluate the FETAX system and to critically test the assumption that FETAX can adequately detect mammalian teratogens. Successful efforts in developing a reliable metabolic activation assay for Xenopus embryos may provide a viable adjunct to in vivo mammalian assays. The need for representative metabolism is considered a requirement for adequate use of in vitro systems in screening potential teratogens (8). A bioactivation system improved the predictive capability as well as enhanced the usefulness of the FETAX test. The assay at present appears capable of detecting over 85% of the teratogens from a moderate group of chemicals (including proteratogens) with few false positives. Since evidence indicates that metabolic activation probably plays a role in teratogenesis (30, 31), much the same as in carcinogenesis, the proposed efforts are indeed significant.

Methods of Approach

1. Validation

We predict that FETAX will perform at the 85% level in terms of its sensitivity and specificity when tested with compounds whose mammalian and human developmental toxicity are known. Controversy does exist about how best to conduct validation studies in this area. The list of compounds published by Smith et al. (22) in 1983 has merit but it has been criticized by many as being too small and weighted toward positives likely to cause teratogenic insult in virtually all species. Additionally, some compounds were listed based on experiments that used routes of administration such as inhalation. We feel this list has been much maligned but that it still as a reasonable starting point for a validation study so long as its limitations are accounted for. Marshall Johnson (32) feels that in vitro teratogenesis testing data should be compared to segment II studies performed with rats. This idea has merit but many of these studies have been performed for the FDA and the results are not made public. Nigel Brown (33) has recommended that testing be done "in the blind" and we have done this for the validation compounds listed in Table 1. Lastly, some believe that the only proper method of comparing the predictive accuracy of validation studies is to perform mammalian tests in conjunction with in vitro tests. Obviously this is the method of choice were it not for the ruinous cost associated with mammalian tests requiring a dose-response effect. In selecting compounds for testing we consider the Smith et al. list, Shepard's "Catalog of Teratogenic Agents" (34), Segment II data gleaned from the literature and any other studies published by good researchers in the field of mammalian teratology. This is a compromise approach but one which we feel has been successful to date. When a compound fails to perform as expected we try the assay again using the rat liver MAS to see if bioactivation or deactivation affected results.

No rules exist as to how many compounds need to be tested before an assay is "acceptable" to regulatory agencies. The Chernoff mammalian teratogenesis screen has been tested with > 150 compounds (See: Teratogenesis, Carcinogenesis and Mutagenesis Vol. 7(1)). FETAX has undergone validation with 90 compounds although the results from 40 compounds have not yet been

published by Dumont because he has left the field (J.N. Dumont personal communication). Dumont did not use the renewal procedure and left the jelly coats on the eggs. These differences in procedure from the ASTM guide necessitate additional testing to further validate the test using the same methodology. The standard FETAX protocol has been provided in section II of the Appendix. Any substantive modifications of this procedure made at future ASTM meetings will be adopted although it is unlikely that this will occur based on the results of the first subcommittee ballot.

Successful validation of FETAX using the compounds proposed will allow its use as an in vitro teratogenesis screening assay. Final acceptance of the assay will depend on a high degree of predictability probably at the 85% level. As of this writing FETAX is now performing at this level.

Section 5 of Results and Conclusions (FETAX Literature) gives the details of the FETAX Assay in ASTM New Standard Guide format.

Solvent Interaction Study

The solvents and test compounds for this study are listed in Table 2. To date triethylene glycol, acetone and dimethyl sulfoxide are among the best candidates to assist in solubilizing nonpolar compounds so that they can be tested. Triethylene glycol is the least toxic and teratogenic but it is not as good a solvent as the other two. Acetone is a good solvent but its TI is slightly high. Dimethyl sulfoxide seems to be the best solvent available. In previous tests, concentrations of solvent at the NOEC had no effect on the activity of the in vitro metabolic activation system employing rat liver microsomes. The four test compounds listed in Table 2 have been used to show how much the inclusion of solvent to the FETAX protocol affects test results. Financial support for finding the 96-hr LC50, 96-hr EC50 (malformation) and MCIG for each compound was provided from another source.

Dose-Response curves for mortality and malformation have been derived for each test compound and solvent. Experiments were conducted by starting at the 96-hr LC25 and EC25 (malformation) for each test compound. One series of experiments were performed at the highest no observable effect concentration (NOEC) for each solvent. A second series of experiments were performed at the 96-hr LC25 and EC25 (malformation) for each solvent and test compound. The appropriate control was the test compound in water only. Positive interactions between the solvent and test compound were observed and were greater than simple additive effects. If there was a negative interaction, then mortality, malformation and growth effects would be less. These negative interactions were observed in this study. For each experiment there were four dishes of 25 embryos each in FETAX solution as controls. Analysis of data and statistics were as previously published for FETAX interaction studies (35).

3. Metabolic activation

We are trying to answer the question of whether we can develop and validate an in vitro metabolic activation system for FETAX using Aroclor 1254 induced rat liver microsomes that closely simulates mammalian metabolism. Although it will not be the same type of metabolic activation

system as found in humans, it should be close enough to detect most mammalian and human proteratogens.

The "Atlas of Abnormalities" (Section 5) details our method of making microsomes and how we standardize the units of P-450 activity. Now that we have developed this method, we are actively engaged in validating the use of these microsomes and fine tuning the procedure for maximum efficiency. We have selected an additional set of 5 compounds to test in this study. Two compounds require activation for a developmentally toxic effect, two are direct-acting teratogens and a third compound appears to be a negative that is not activated (Table 3). Our plan is to perform two experiments on each compound with and without in vitro metabolic activation system. Because of the complexity of these experiments only 20 embryos per dish were used instead of the standard 25. Dose-response curves for mortality and malformation were compared as well as Teratogenic Indices (TIs). The relative changes in the 96-hr LC and EC50s indicated whether any bioactivation or inactivation occurred. Similarly, growth curves can be compared to determine whether metabolic activation was required. Compounds listed on Table 1 were tested using the in vitro MAS if they did not perform as expected in the standard FETAX assay. In these cases tests were run only at the direct-acting 96-hr LC and EC50 concentrations. Increases or decreases from the 50% median effect levels (mortality and malformation) served as an indication as to whether the microsome-treated embryos differed from controls due to metabolism.

4. Atlas of Abnormalities

The manual will be provided to the Army, Army contractors and other individuals gratis until the supply is exhausted. Postage and handling charges will be the only cost borne by the new user. No royalties will accrue to the PI.

The manual covers mating the frogs, staging of embryos, diseases of adult frogs and their remedies and, lastly, all the major malformations. Particular emphasis was placed and judging slight malformations as this affects the scoring of the malformation endpoint the greatest. We saved all the embryos from each experiment and have gone through them looking for each different type of malformation. Color negatives were made and all of the negatives were first printed in black and white. We then arranged the best micrographs and selected which need to be presented in color and which were presented in black and white. These micrographs were then pasted up and the legends added. Dr. James Dumont and our COR, Dr. Robert Finch, then commented on each chapter. After revisions, the Atlas was peer-reviewed by the Army. Finally, it was sent to the publications department of Oklahoma State University for processing and printing.

5. Comparative Sensitivity of FETAX

In this study, we compared FETAX with a modified fathead minnow assay. Previous comparisons of FETAX with other amphibian and fish assays were all faulted by differing environmental conditions. However, in order for FETAX to be compared with the fathead minnow assay, we had to lengthen the test to 5 days. We also had to establish a day/night cycle so that the minnows would hatch. We carefully controlled all environmental parameters. Three

compounds were tested. Each of these had previously been tested in the four day version of FETAX so we knew which concentrations to use. We could also assess whether the 5 day TI decreased from the 4 day TI (it did). Although other fish species are known to be more sensitive than fathead minnows, we feel that FETAX will be at least as sensitive as most other vertebrates in use today.

Table 1. List of Test Compounds used for FETAX Validation.

| Compound | CAS # | MMS | Teratogen* | Solu- bility *** | Range Finder/ Definitive 1 LC50 EC50 TI** | Definitive 2 LC50 TI | Definitive 3 LC50 EC50 TI |
|---------------------------------------|------------|--------|------------|---------------------|---|-------------------------------|-------------------------------|
| <u>Validation Compounds-Phase I</u> | | | | | | | |
| Anaranth **** (mg/ml) | 915-67-3 | N | - | 1 | 2.67 3.5 0.8 (MCIG 4.0) | 3.68 3.1 1.2 (MCIG>3.0) | 3.81 3.91 0.97 (MCIG 3.75) |
| Aspartame (mg/ml) | 22839-47-0 | N | - | 3 | >8.0 >8.0 NA (MCIG 3) | >10.0>10.0 NA (MCIG 7) | 13.9 13.1 1.1 (MCIG 7) |
| 5-Azacytidine (mg/ml) | 320-67-2 | N | + | 1 | 0.59 0.014 42 (MCIG 0.04) | 0.43 0.02 21 (MCIG 0.07) | 0.6 0.07 9 (MCIG 0.1) |
| Methotrexate**** | 59-05-2 | N | + | 1 | R 0.79 0.02 43 (MCIG 0.05) | 0.51 0.02 23 (MCIG 0.02) | 0.5 0.03 17 (MCIG 0.01) |
| d-Pseudoephedrine HCl (mg/ml) | 345-78-8 | N | - | 2 | 0.44 0.26 1.7 (MCIG 0.2) | 0.42 0.23 1.8 (MCIG 0.2) | 0.39 0.21 1.9 (MCIG 0.15) |
| d-Pseudoephedrine HCl (mg/ml)***** | 345-78-8 | N Y | - - | 2 2 | ca.0.43 0.21 NA >0.43 >.43 NA | 0.43 0.23 NA >0.43 >.43 NA | NA >.43 NA |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

***** A limited test with rat liver microsomes was conducted with pseudoephedrine. Only a limited number of concentrations were used. In the presence of microsomes, there was no observable toxicity or teratogenicity.

Table 1 continued. List of Test Compounds used for FETAX Validation.

| Compound | CAS # | MAS | Teratogen* | Solubility *** | Range Finder/Definitive 1 IC ₅₀ EC50 TI** | Definitive 2 IC50 EC50 TI | Definitive 3 IC50 EC50 TI |
|---------------------------------------|------------|-----|------------|----------------|---|---|------------------------------|
| <u>Validation Compounds- Phase II</u> | | | | | | | |
| Ascorbic acid (mg/ml) | 50-81-7 | N | - | 1 | 19.2 11.6 1.7 (MCIG 10.0) | 20.3 12.8 1.6 (MCIG 10.0) | 19.6 12.0 1.6 (MCIG 10.0) |
| Sodium selenate (ug/ml) | 13410-10-0 | N | V(+) | 2 | 17.0 6.0 3 (MCIG 14) | 19.0 7.0 2.8 (MCIG 6) | 27.0 9.0 3.1 (MCIG 8) |
| Coumarin (mg/ml) | 91-64-5 | N | V(+) | 2 | 0.15 0.038 4 (MCIG 0.01) | 0.14 0.038 3.50.1 (MCIG 0.05) | 0.045 2 (MCIG 0.04) |
| Serotonin (mg/ml) | 153-98-0 | N | + | 2 | 2.74 0.35 | 7.8 3.27 0.39 8.4 3.21 0.48 6.7 (MCIG 0.25) (MCIG 0.6) | (MCIG 1.0) |
| 13-cis Retinoic Acid (ng/ml) | 4759-48-2 | N | + | 4 | 37.0 3.0 18.8 (MCIG 19) | 18.0 2.0 9.2 36.0 4.0 10.1 (MCIG NA) | (MCIG 28) |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

Table 1 continued. List of Test Compounds used for FETAX Validation.

| Compound | CAS # | MAS | Teratogen* | Solu- bility *** | Range Finder/ Definitive 1 IC50 EC50 TI** | Definitive 2 TI | Definitive 3 EC50 TI |
|--|----------|-----|------------|---------------------|---|------------------------------|------------------------------|
| <u>Validation Compounds- Phase III</u> | | | | | | | |
| <u>Solvents</u> | | | | | | | |
| Triethylene Glycol (% v/v) | 112-27-6 | N | - | 1 | 2.4 2.0 1.2 (MCIG 1.8) | 2.75 2.4 1.1 (MCIG 1.8) | 2.19 2.05 1.07 (MCIG 1.7) |
| Acetone (% v/v) | 67-64-1 | N | - | 2 | 2.16 1.4 1.6 (MCIG 1.25) | 2.49 1.4 1.8 (MCIG 1.5) | 1.92 1.06 1.83 (MCIG 1.0) |
| Dimethyl sulfoxide (% v/v) | 67-68-5 | N | V(-) | 1 | 1.81 1.4 1.3 (MCIG 1.25) | 1.77 1.29 1.4 (MCIG 1.7) | 1.86 1.24 1.5 (MCIG 1.2) |
| <u>Teratogens</u> | | | | | | | |
| trans-Retinoic Acid (ug/ml) | 302-79-4 | N | + | 4 | 0.25 0.024 10.4 (MCIG 0.06) | 0.5 0.44 11.4 (MCIG 0.08) | |
| Me-mercury chloride (mg/ml) | 115-09-3 | N | + | 1 | 0.083 .024 3.40.09 (MCIG 0.036) | 0.025 3.7 (MCIG 0.04) | |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

Table 1 continued. List of Test Compounds used for FETAX Validation.

| Compound | CAS # | MAS | Teratogen* | Solubility *** | Range Finder/ Definitive 1 | Definitive 2 | Definitive 3 |
|--------------------------------|----------|-----|------------|----------------|-------------------------------|-----------------------------|--------------|
| | | | | | IC50 EC50 TI** | IC50 EC50 TI | IC50 EC50 TI |
| Validation Compounds- Phase IV | | | | | | | |
| Busulfan (mg/ml) | 55-98-1 | N | - | 2 | R0.2 0.161.3 (MCIG NA) | | |
| Furazolidone (mg/ml) | 67-45-8 | N | + | 2 | 15.0 12.0 1.3 (MCIG 9.0) | 14.0 7.11 2.0 (MCIG 8.0) | |
| Procarbazine (mg/ml) | 671-16-9 | Y | + | 2 | 1.69 NA (MCIG 1.25) | 3.17 1.31 2.4 (MCIG 1.0) | |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

Table 2. List of Proposed Test Compounds for Determining Possible Solvent Interaction Effects.

| Compound | CAS # | MAS | Teratogen* | Solu- bility *** | Range Finder/ Definitive 1 | Definitive 2 | Definitive 3 |
|--|----------|-----|------------|---------------------|-------------------------------|--------------------------------|------------------------------|
| <u>Solvents</u> | | | | | | | |
| Triethylene Glycol (% v/v) | 112-27-6 | N | - | 1 | 2.4 2.0 1.2 (MCIG 1.8) | 2.75 2.4 1.1 (MCIG 1.8) | 2.19 2.05 1.07 (MCIG 1.7) |
| Acetone (% v/v) | 67-64-1 | N | - | 2 | 2.16 1.4 1.6 (MCIG 1.25) | 2.49 1.4 1.8 (MCIG 1.5) | 1.92 1.06 1.83 (MCIG 1.0) |
| Dimethyl sulfoxide (% v/v) | 67-68-5 | N | V(-) | 1 | 1.81 1.4 1.3 (MCIG 1.25) | 1.77 1.29 1.4 (MCIG 1.7) | 1.86 1.24 1.5 (MCIG 1.2) |
| <u>Compounds used in Solvent Interaction Study (t-retinoic acid & Me-mercury chloride on Table 1).</u> | | | | | | | |
| Trichloroethylene (% v/v) | 79-01-6 | N | - | 4 | 0.024 0.048 5 (MCIG >0.08) | .029 0.002 12.6 (MCIG 0.02) | |
| **** | | | | | | | |
| 6-aminonicotinamide (ng/ml) | 329-89-5 | N | + | 1 | 3.19 .053 602 | 2.95 .057 518 | |
| **** | | | | | | | |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983. Supported by OCAST.

Table 3. List of Proposed Test Compounds for FETAX Metabolic Activation System Validation.

| Compound | CAS # | MAS | Teratogen* | Solubility *** | Range Finder/ Definitive 2 | Definitive 3 |
|---|----------|-----|------------|--|---|--------------|
| | | | | | LC50 EC50 TI**LC50 EC50 TI | LC50 EC50 TI |
| <u>Validation Compounds for Metabolic Activation System</u> | | | | | | |
| Acetaminophen (mg/ml) | 103-90-2 | ? | V(-) | 2 NO MAS | 0.15 0.13 1.1 0.19 0.13 1.5>3.0 0.1 >3.0 (MCIG 0.1) (MCIG <.12) (MCIG 0.15)***** | |
| Acetazolamide **** (mg/ml) | 59-66-5 | N | V(+) | 3 R NO MAS>0.1 >0.1 ***** | | |
| Benzo(a)pyrene (ug/ml) | 50-38-2 | Y | + | 4 MAS >10.0 0.2>5 >10.0 1.5>6.7>10.0 1.8 >5.6 (MCIG 0.5) (MCIG 0.5) | | |
| | | | | NO MAS >10.0 10.0>1 >12.0 10.0>1.2>10.0 10.0 >1.0 (MCIG 1) (MCIG 5) | | |
| Dimethylnitrosamine (mg/ml) | 62-75-9 | Y | + | 2 NO MAS 3.5 2.3 1.6 3.2 2.3 1.4 MAS 2.6 2.0 1.3 | | |
| Sodium Salicylate (mg/ml) | 54-21-7 | N | V+ | 1 MAS 2.2 1.4 1.6 2.3 1.5 1.5 (MCIG <1.2) (MCIG <1.2) | | |
| | | | | NO MAS 2.34 1.7 1.4 2.3 1.23 1.87 | | |

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.

** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.

*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.

**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

***** Limit of solubility in 1% DMSO.

***** with penicillin-streptomycin.

Forword to the Validation Section

We have divided the validation section into four phases. Each phase was performed by a different investigator who worked in Dr. Bantle's laboratory. We kept the data separate in case it became necessary to reanalyze data at a later date due to possible variation in judging abnormalities. Phase I was performed by P.K. Work with help from Shirley Bush. Phase II was conducted by Donna DeYoung and Phase III by James Rayburn. Phase IV was performed by Lynne Homer with help from Mendi Hull. Although the investigator was different, the methodology employed was the same throughout the study. Typical raw data summary sheets are provided at the end of the validation section.

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Results

1. Validation

A.) Phase I

Table 1 shows the validation data collected to date. When a compound is tested in FETAX, range finding experiments are first conducted in order to determine the best concentrations of toxicant to use in establishing mortality and malformation dose-response curves. The results of these tests are not present in Table 1. After the proper concentration ranges are selected, then at least three definitive tests are performed in the blind. In practice, Mr. Doug Fort labeled the compounds A, B, C, etc. and handed them to the technician who then conducted the assay. The technician worked under high hazard containment at all times as she did not know the identity of the test compound. It must be remembered that the dose-response curves presented only show the data points (50 embryos per data point) used in constructing the dose-response curve. In practice there were many additional concentrations tested in each experiment.

The compounds selected for testing were Amaranth, Aspartame, 5-Azacytidine, Methotrexate and d-Pseudoephedrine. Based on the mammalian literature, 5-Azacytidine and Methotrexate were thought to be positives while the other compounds were thought to be negatives. In the case of Pseudoephedrine, the in vitro metabolic activation system was used because results were not as anticipated. This data and its comparison to controls is listed in the last row of Table 1.

Amaranth: Amaranth (Red food dye #2) is a compound listed by Smith et al. (22). It has generally been shown to be a nonteratogen in mammalian and human studies. Shepard (34) lists 4 studies on Amaranth with only one report dissenting from the conclusion that Amaranth is not a developmental toxicant. Amaranth is compound that has been independently tested in FETAX before by both Sabourin and Faulk (14) and James Dumont (Oak Ridge National Labs-personal communication). They found that Amaranth was a negative as well. We repeated their work with this compound in order to estimate the reliability and repeatability of FETAX and to detect any co-validation problems early on with FETAX. It must be remembered that both Sabourin and Faulk and Dumont both performed FETAX slightly differently than the ASTM Guide.

Table 1 shows that Amaranth is clearly not a developmental toxicant. The average TI was 0.99 and the MCIG (minimum concentration that statistically inhibits growth at $P=0.05$) averaged 3.58. This was even greater than the average LC50 of 3.39. Development toxicants generally inhibit growth at concentrations far less than the 96-hr LC50. Figures 1, 3 and 5 show the dose-response curves for all three definitive experiments. While not superimposable, it is clear that the results pointed to the same conclusion. Figures 2, 4 and 6 show that there is little effect on growth regardless of the concentration used. Plates 1A and 1B show micrographs of larvae exposed to Amaranth continuously for 96 hr. Plate 1 is blurred slightly because this micrograph was taken before we got a vibration mount for our photomicroscope. Nonetheless, the micrographs show that only concentrations well above the 96-hr LC50 cause malformation. Strong teratogens affect development at concentrations far below the 96-hr LC50. Even at high Amaranth concentrations, the malformation are not severe. We

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conclude that Amaranth was not a developmental toxicant in FETAX and this agrees with the vast majority of mammalian literature.

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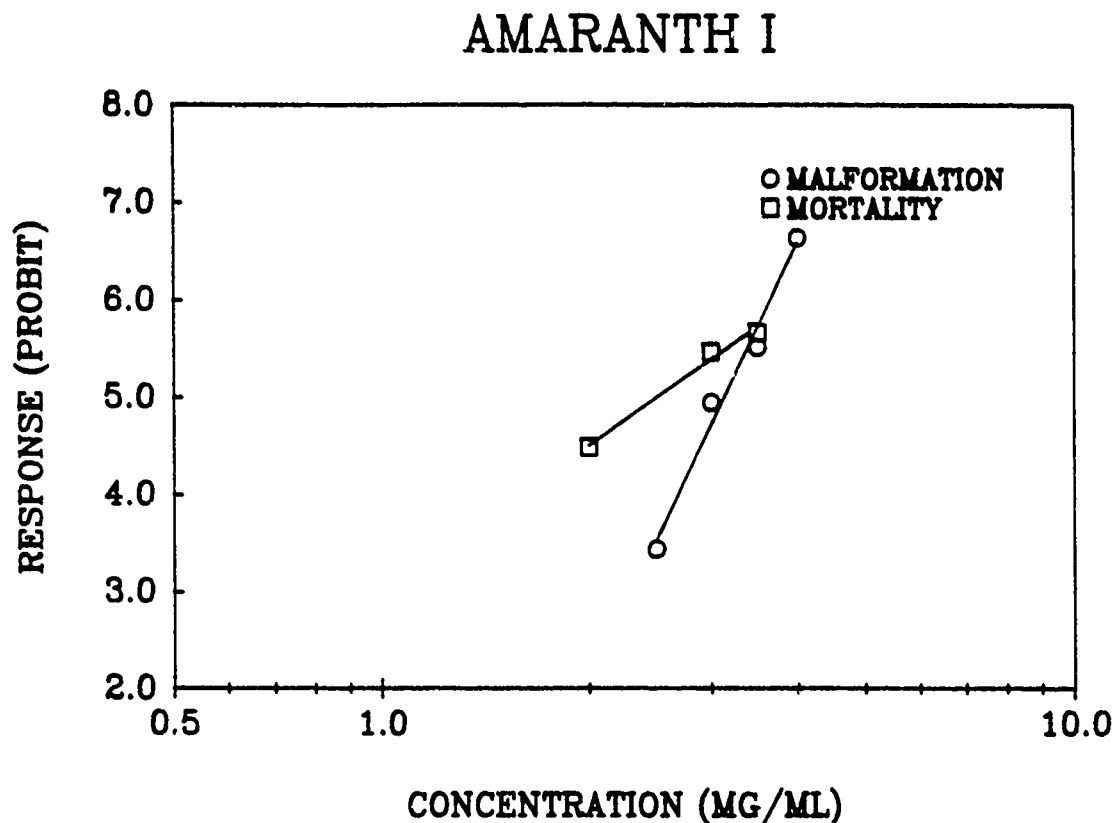


Figure 1. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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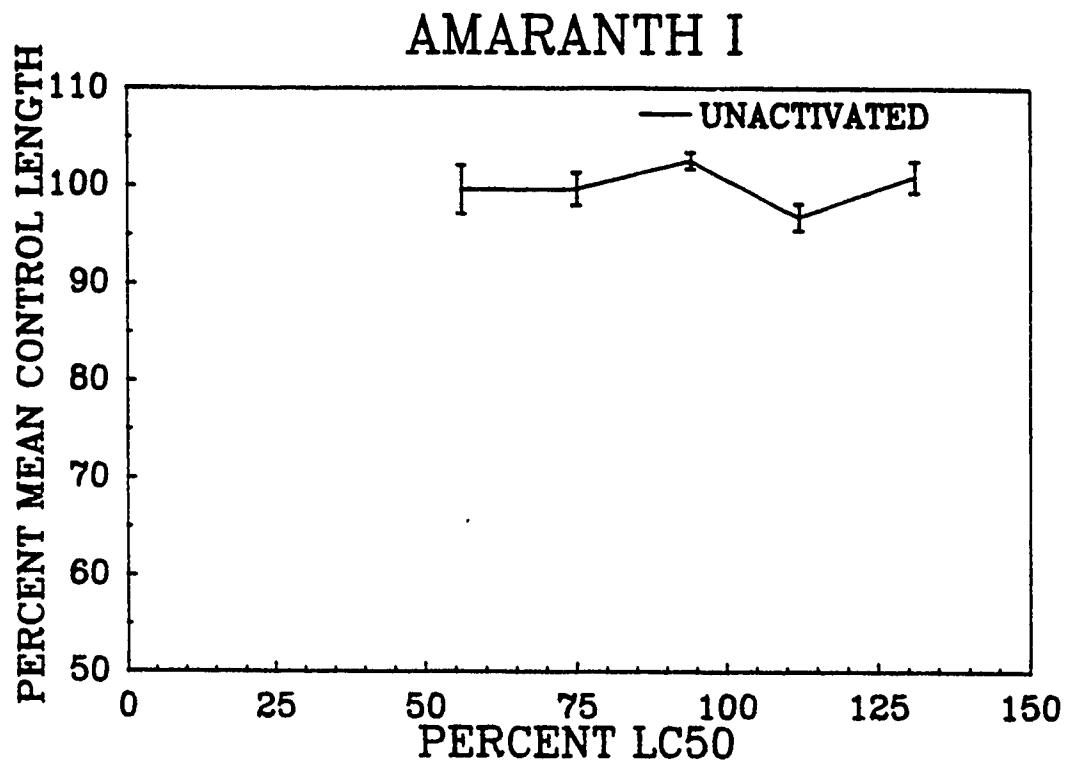


Figure 2. 96-h Growth Dose-Response Curve for Amaranth, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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AMARANTH II

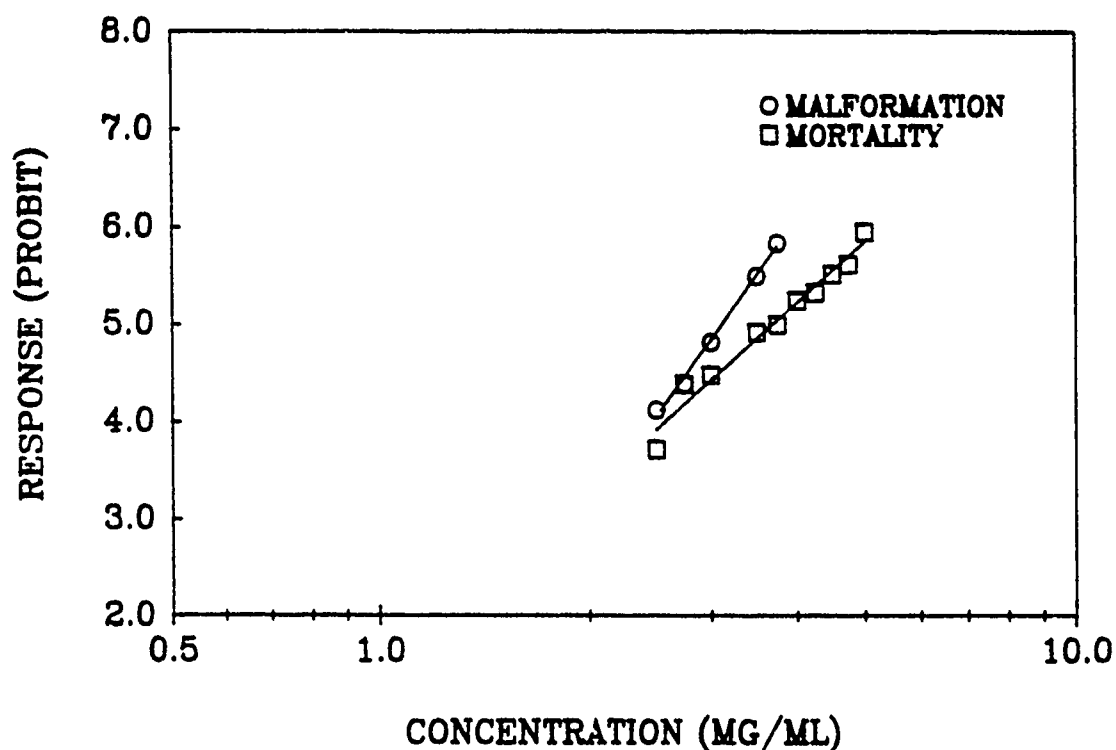


Figure 3. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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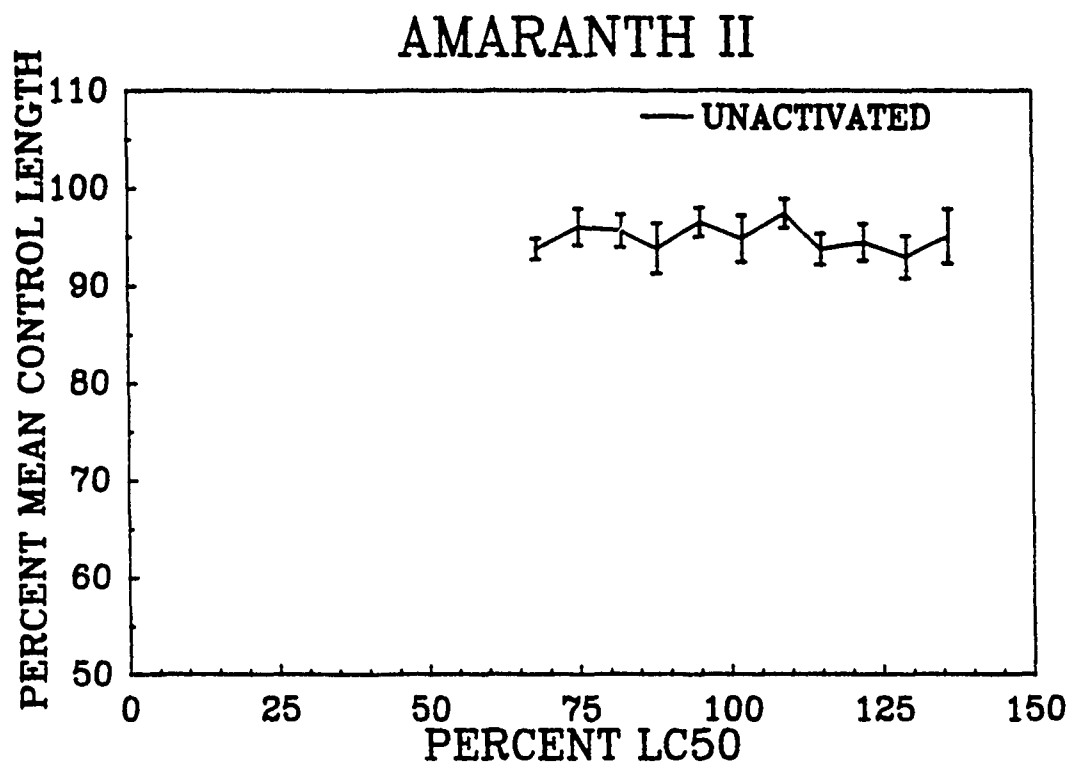


Figure 4. 96-h Growth Dose-Response Curve for Amaranth, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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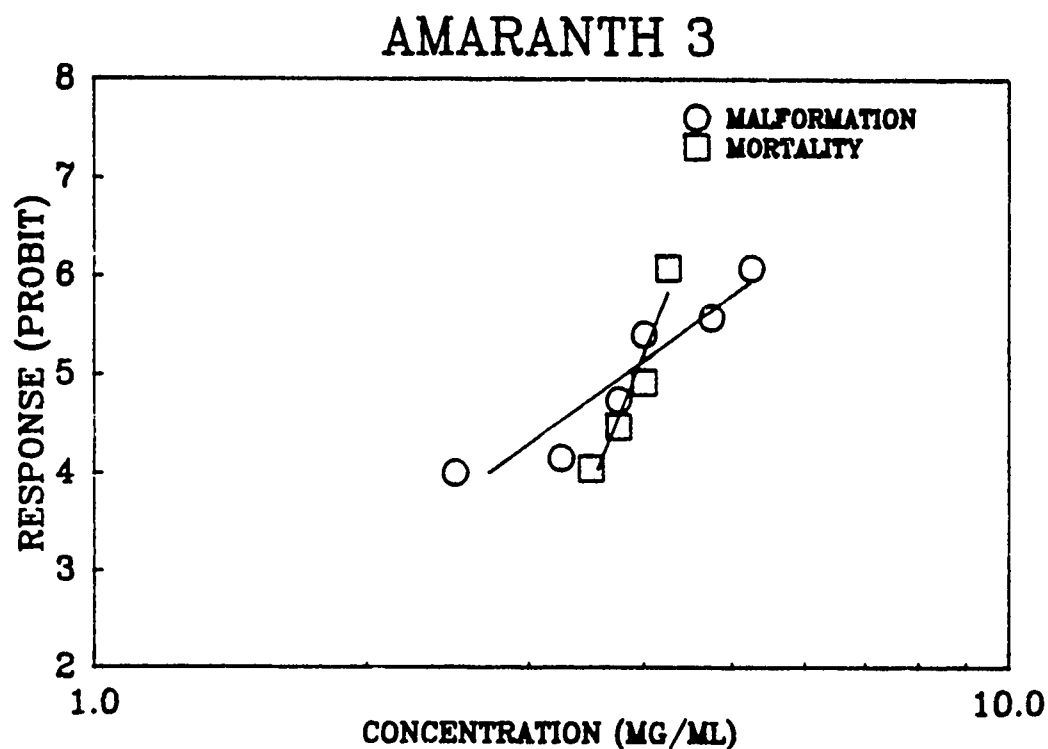


Figure 5. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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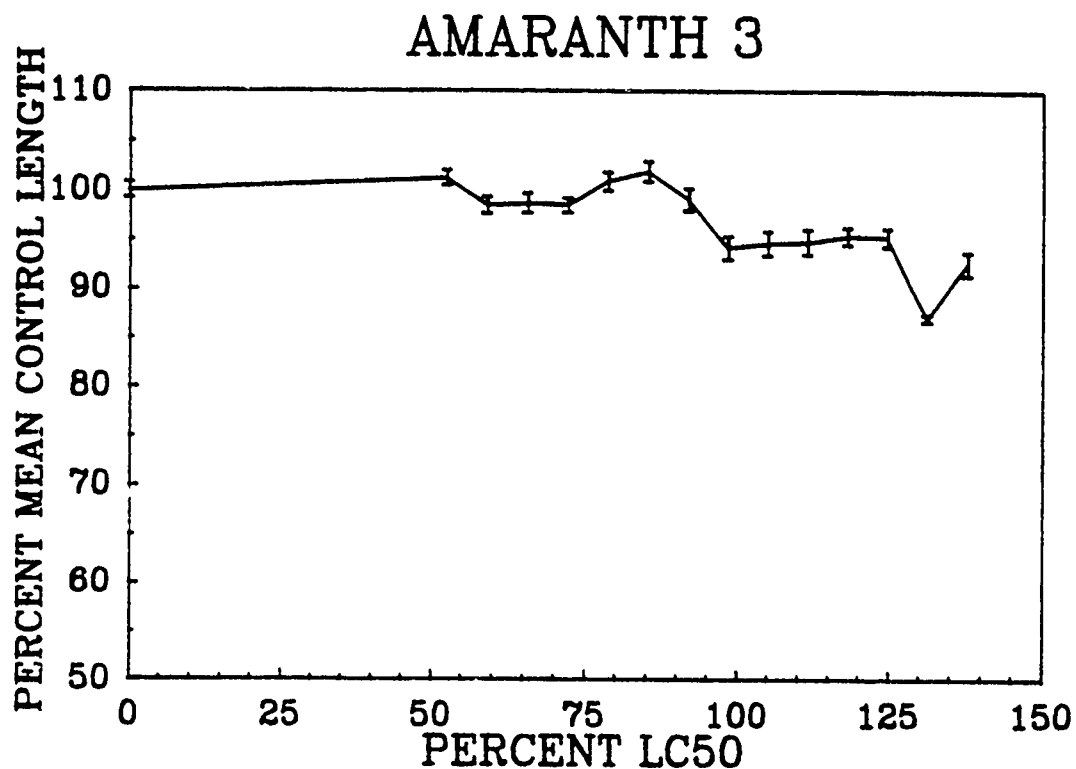


Figure 6. 96-h Growth Dose-Response Curve for Amaranth, Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 1A. Effects of Different Concentrations of Amaranth on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on gut coiling. From top to bottom: control, 2.5 mg/ml, 4.25 mg/ml, 5 mg/ml.



Plate 1B. Effects of Different Concentrations of Amaranth on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2.5 mg/ml, 4.25 mg/ml, 5 mg/ml.

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Aspartame: (Nutrasweet- an artificial sweetner) is another water soluble compound that is not a developmental toxicant (34). It is not on the Smith list. Table 1 shows that it was essentially nontoxic even at concentrations approaching its limit of solubility in water. In the third definitive experiment, we were able to derive a TI of 1.1 for Aspartame by extrapolating the data to the 50% effect levels. This result is compatible with the shapes of the mortality and malformation dose-response curves seen in Figures 7, 9 and 11. The slope of the dose-response curve is very gradual and the two curves are very close to one another. All three growth-inhibition curves (Figs. 8, 10 and 12) are consistent and suggest that while growth inhibition with Aspartame is greater than Amaranth, it is still not a serious growth inhibitor. Plate 2B shows that Aspartame does cause more severe malformations than Amaranth. However, it does take high concentrations around 7-8 mg/ml to cause these malformations. When they occur, virtually all organ systems of the body are affected with equal frequency. Rupture of the eye is common at the higher concentrations. Cardiac and gut malformations are common over wide concentration ranges but curvature of the spine seems to occur only at the highest concentrations. Aspartame is still a negative because of its low TI and its limited effect on development but it is more embryotoxic, teratogenic and growth inhibiting than Amaranth.

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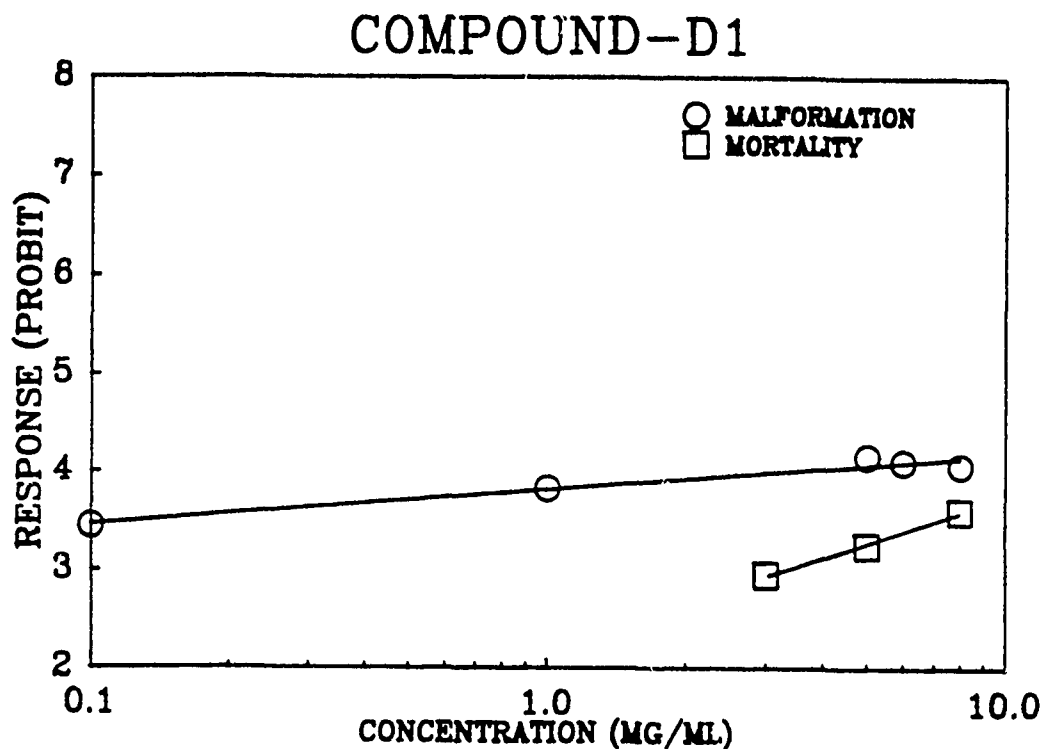


Figure 7. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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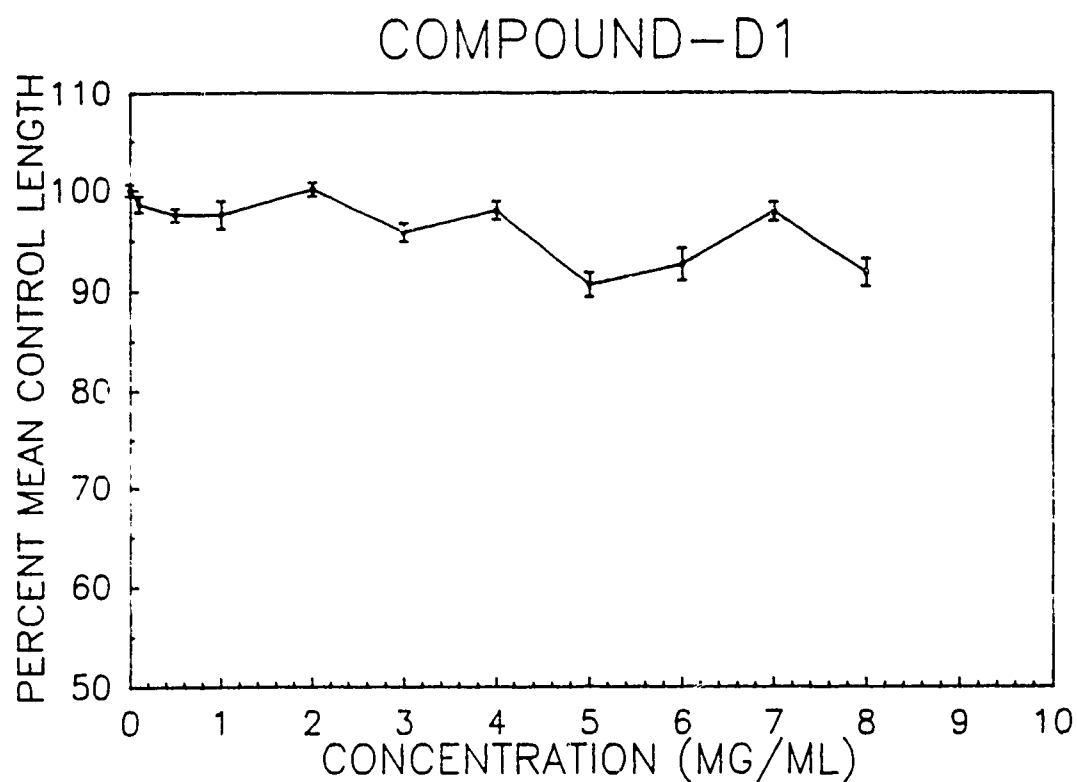


Figure 8. 96-h Growth Dose-Response Curve for Aspartame Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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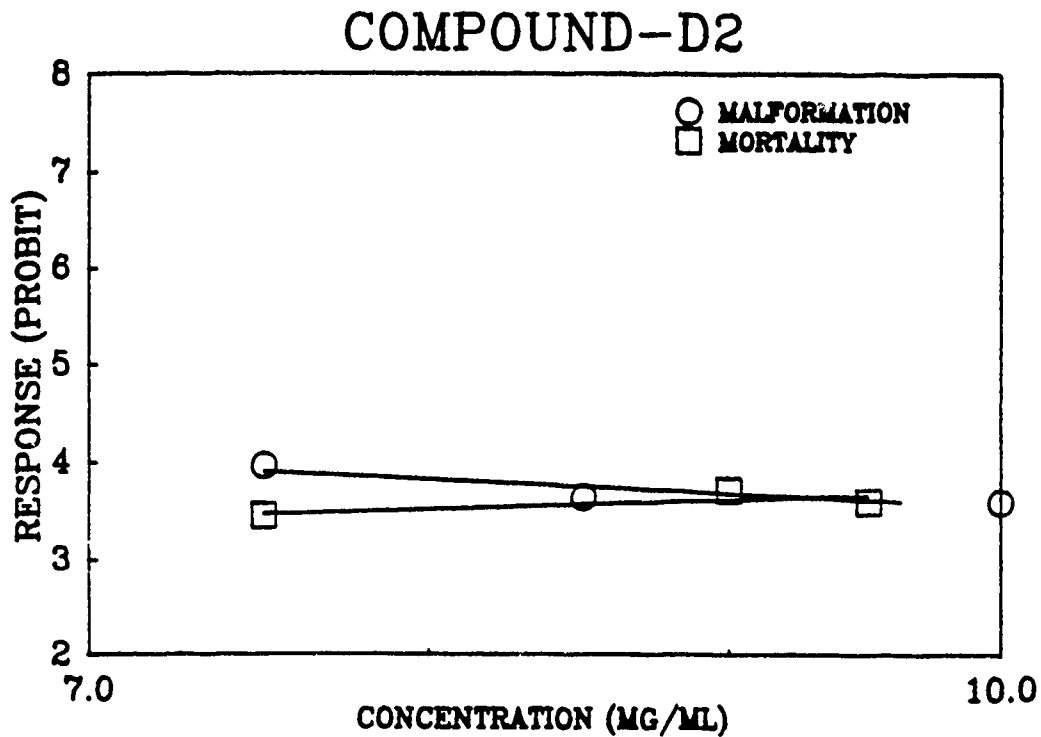


Figure 9. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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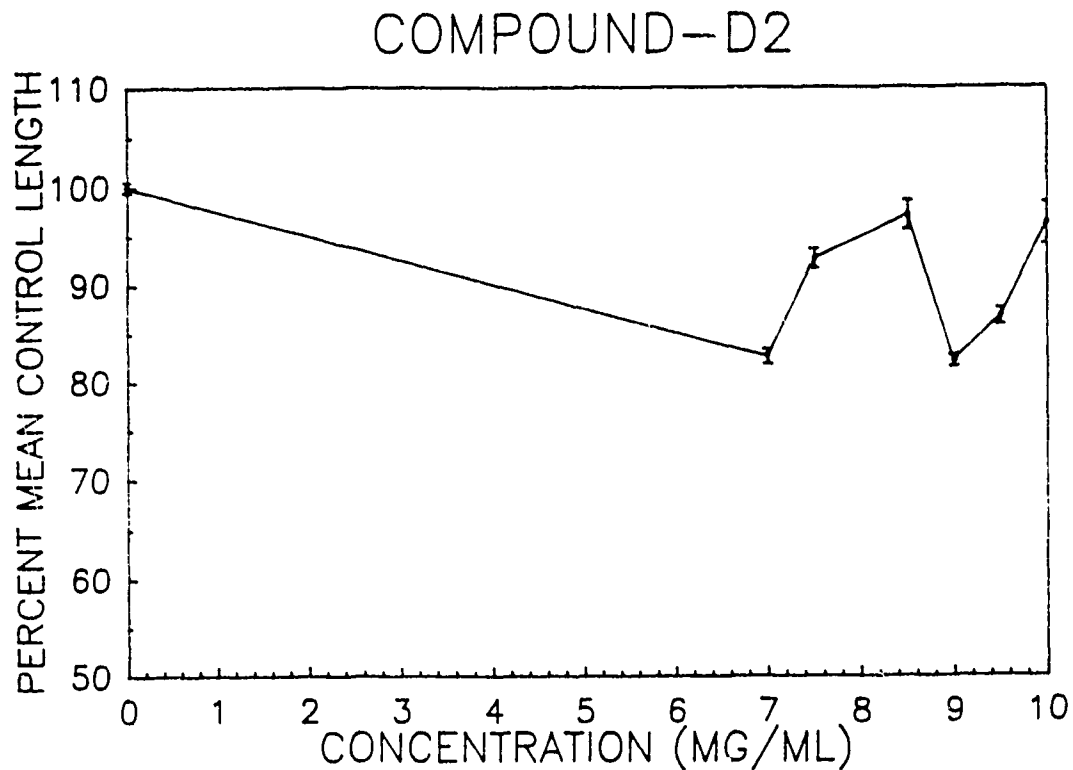


Figure 10. 96-h Growth Dose-Response Curve for Aspartame Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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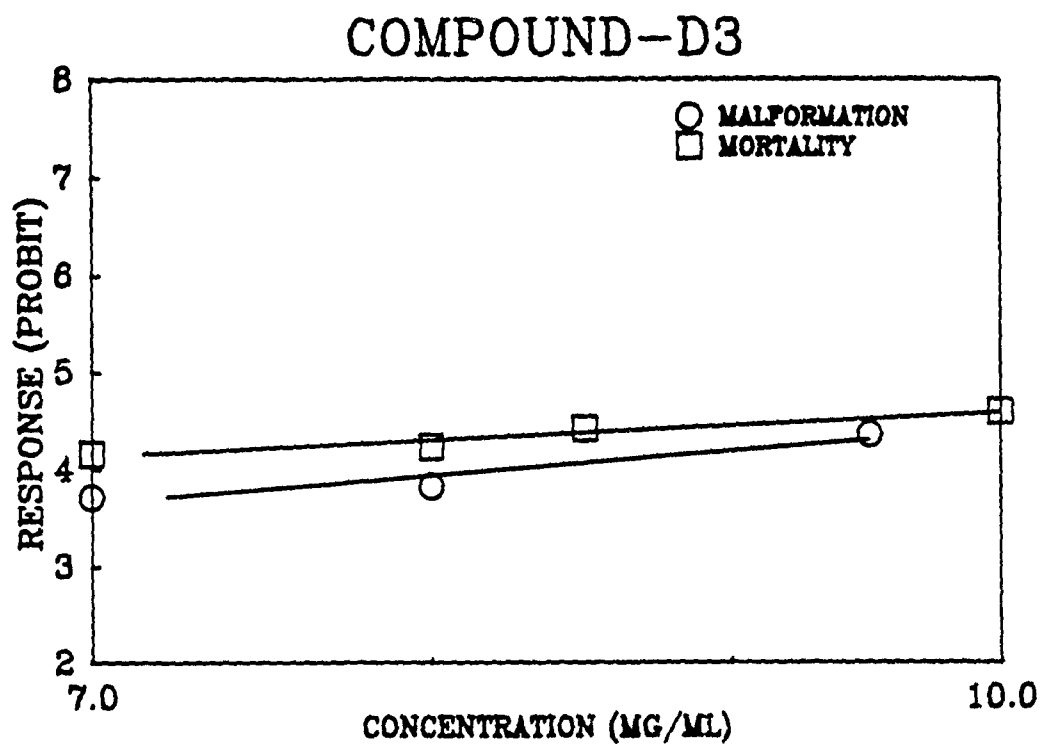


Figure 11. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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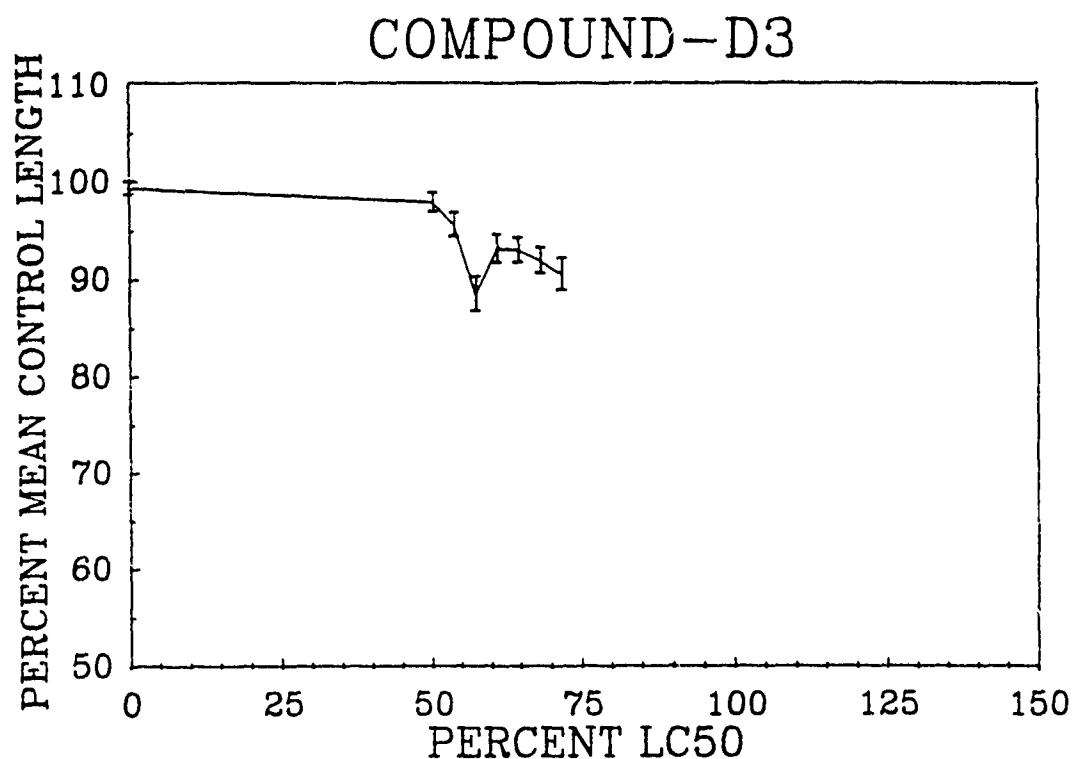


Figure 12. 96-h Growth Dose-Response Curve for Aspartame Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

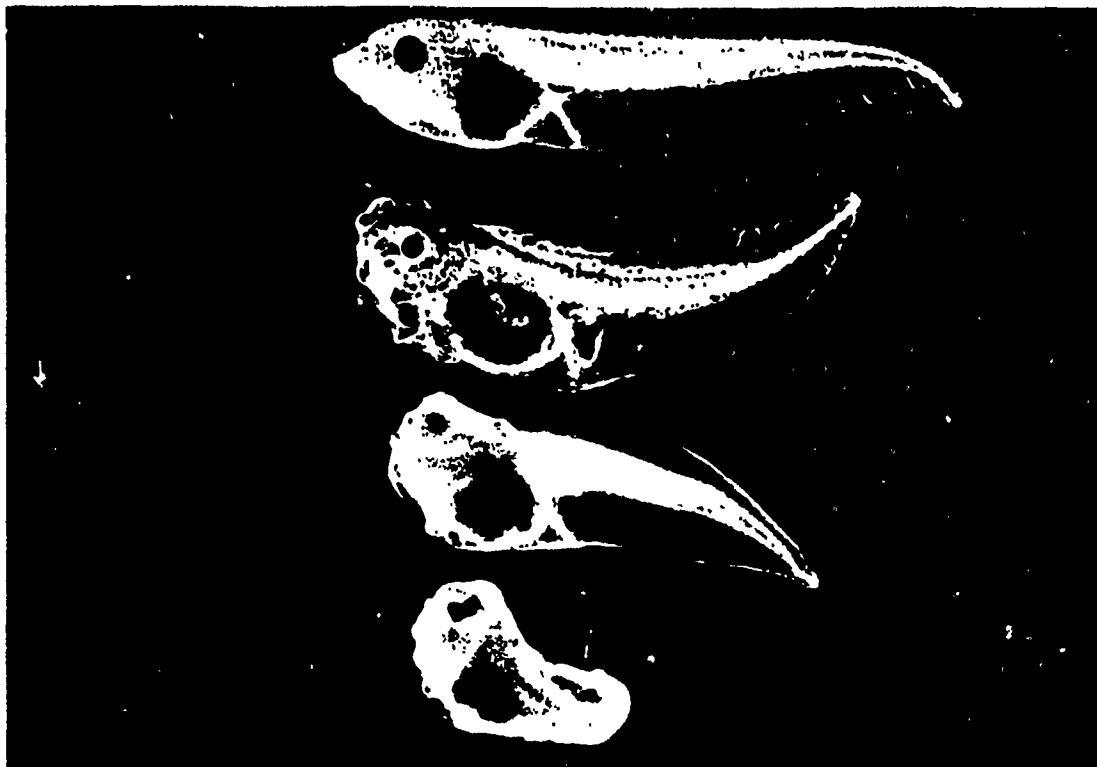


Plate 2B. Effects of Different Concentrations of Aspartame on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, medium concentration, high concentration and very high concentration (near total dead).

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5-Azacytidine: 5-Azacytidine is a potent teratogen (34, 36) because it is an inhibitor of nucleic acid synthesis (9). It is not on the Smith list but it causes effects similar to hydroxyurea and 5-Fluorouracil. The latter two compounds are on the Smith list as strong developmental toxicants in animals. We did have some difficulty in performing definitive tests with this compound. Table 1 shows that we were consistent in obtaining 96-hr LC50 values but that we did have problems scoring malformations at certain concentrations and that there was some variability in effect for this endpoint. The TI ranged from a low of 9 to a high of 42 indicative of a strong teratogen (Table 1). Figures 13, 15 and 17 show mortality and malformation dose-response curves for 5-Azacytidine. The mortality curve is clearly separate from the malformation curve and the fit of the data points is excellent. This separation of the curves indicates the teratogenicity of 5-Azacytidine. The effect of 5-Azacytidine on growth is shown in Figures 14, 16, and 18.

The growth inhibition curves shown in Figs. 16 and 18 are nearly identical while Fig 14. is still consistent with the other two graphs. All suggest that 5-Azacytidine is a strong teratogen because there is a significant growth reduction at concentrations that are less than 20% of the 96-hr LC50, a sharp slope in the curve and a greater than 20% decrease in body length at the highest concentrations. All of these are good indicators of a strong developmental toxicant that can inhibit growth.

Plates 3A-5B show the effect of 5-Azacytidine on embryo development. All major organ systems seem to be equally involved and the malformations even at relatively low concentrations (Plate 3B-0.06 mg/ml). There are few eye malformations and pigmentation of the eye appears normal. There may be a slight tendency towards failure of the ventral choroid fissure to fuse. There is a size reduction of the eye but it is correlated to body length so it is not abnormal. There is a consistent upward bend to the tail (Plates 3A, 4B and 5A). The tendency towards severe malformations being caused at low concentrations of toxicant is consistent with the hypothesis that 5-Azacytidine is a strong teratogen.

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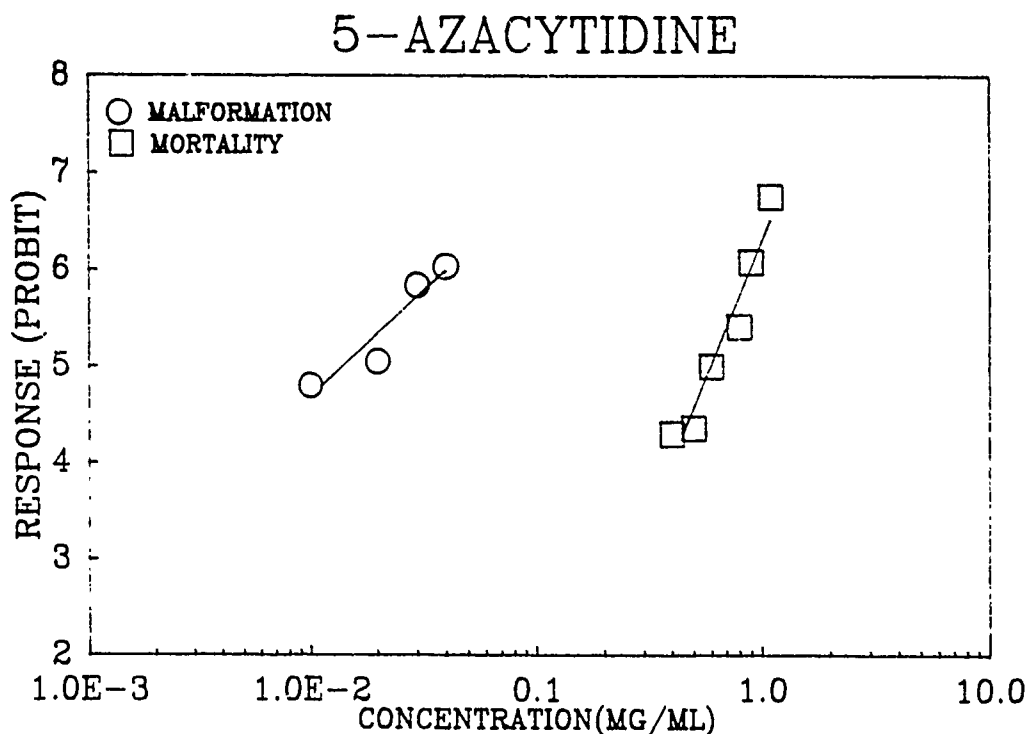


Figure 13. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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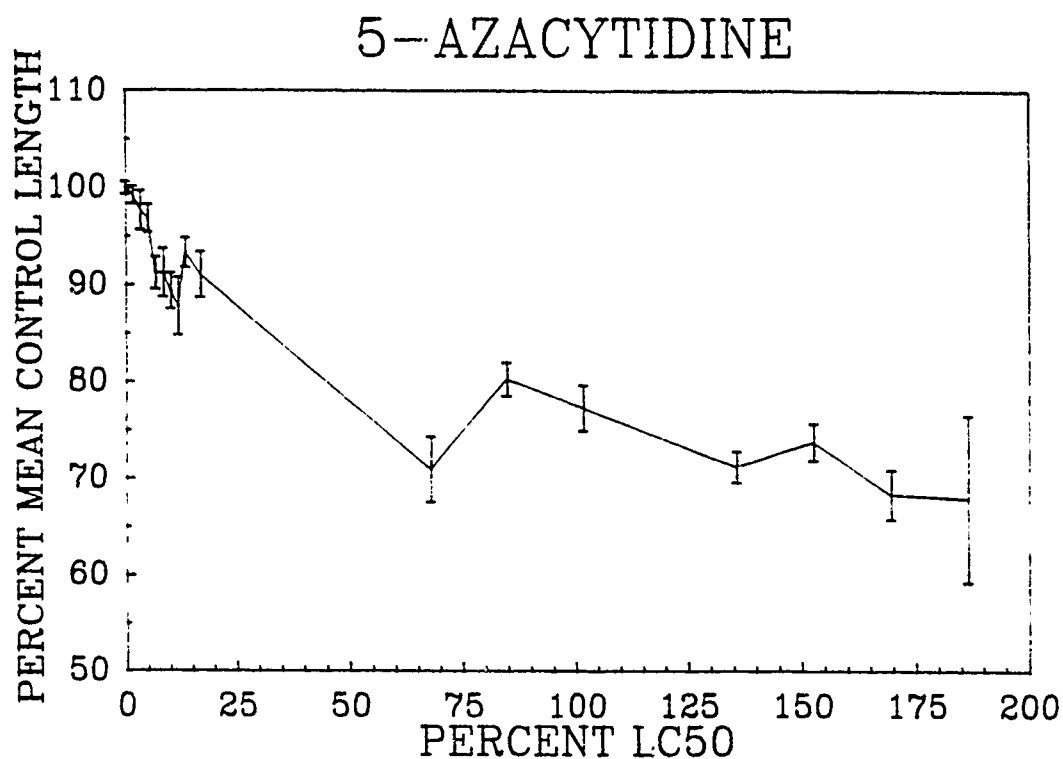


Figure 14. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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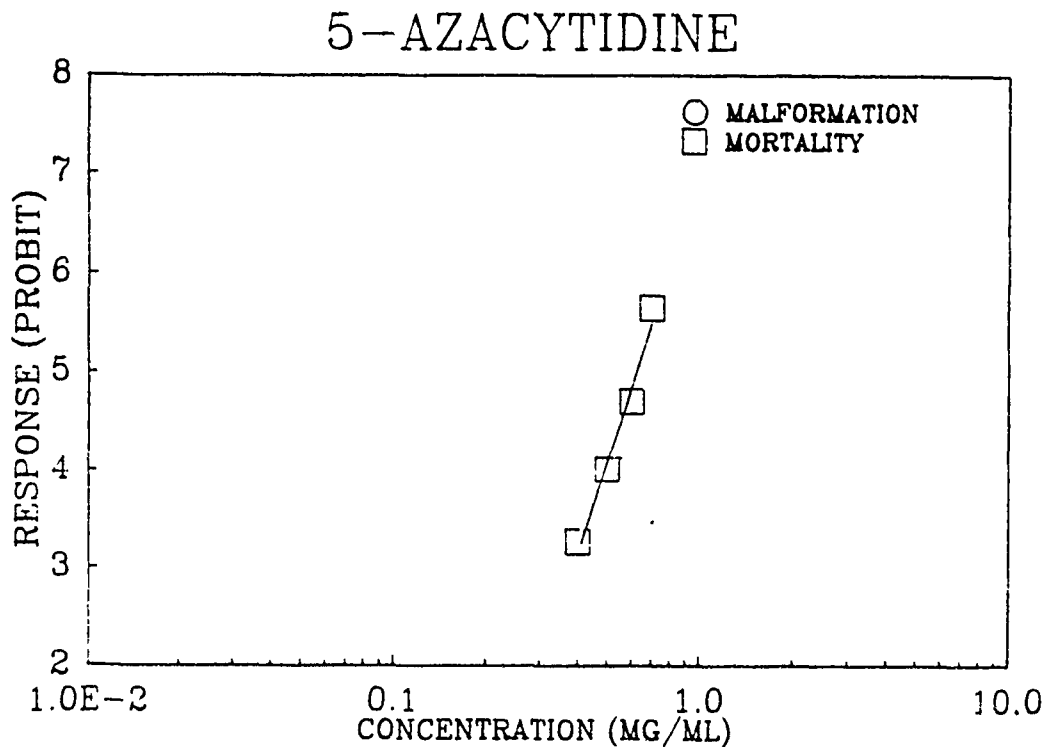


Figure 15. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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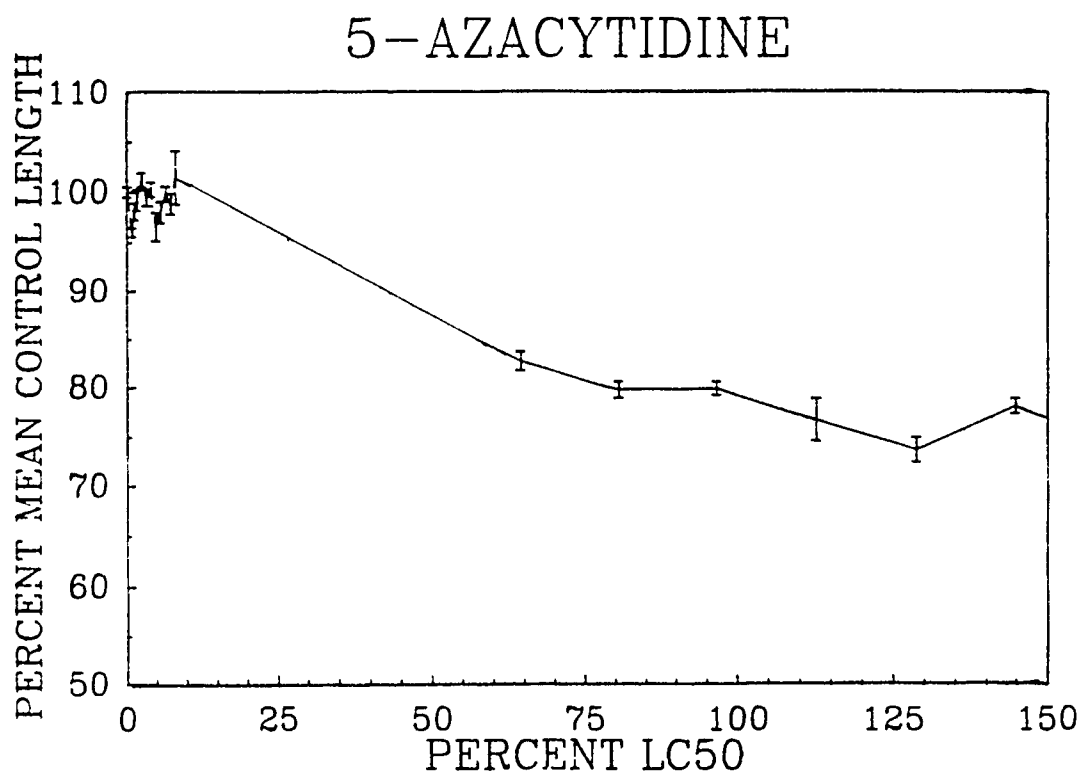


Figure 16. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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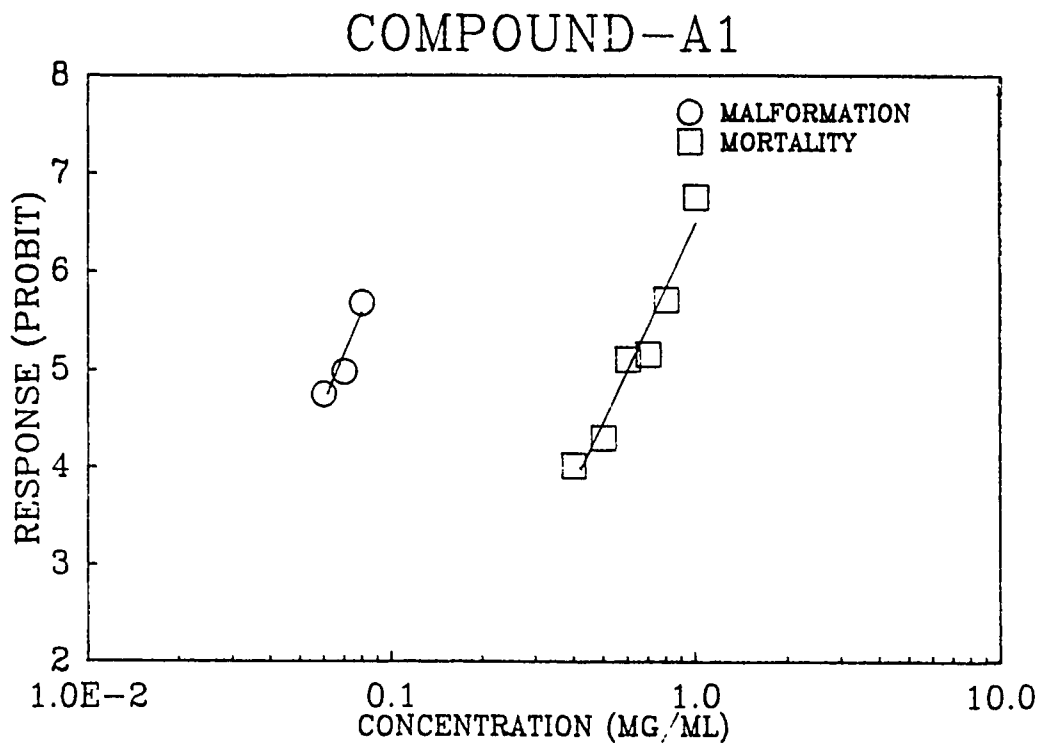


Figure 17. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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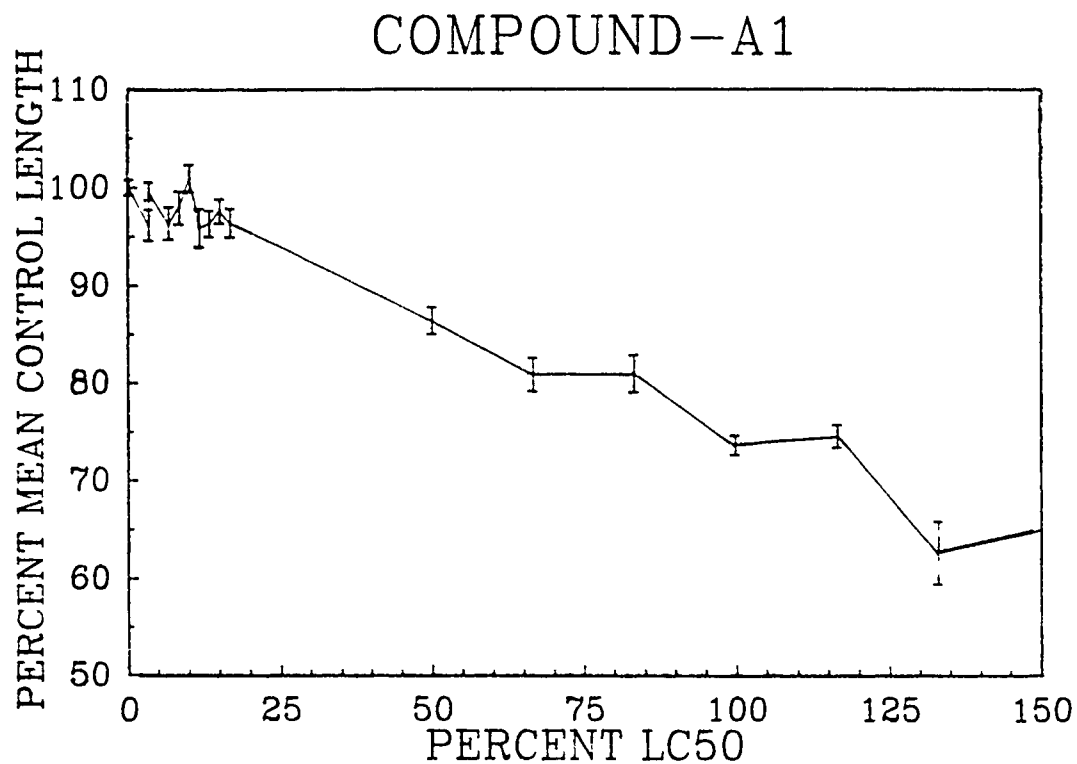


Figure 18. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 3A. Effects of Different Concentrations of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.06 mg/ml, 0.3 mg/ml, 0.8 mg/ml.



Plate 3B. Effects of a Low Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.06 mg/ml 5-Azacytidine.

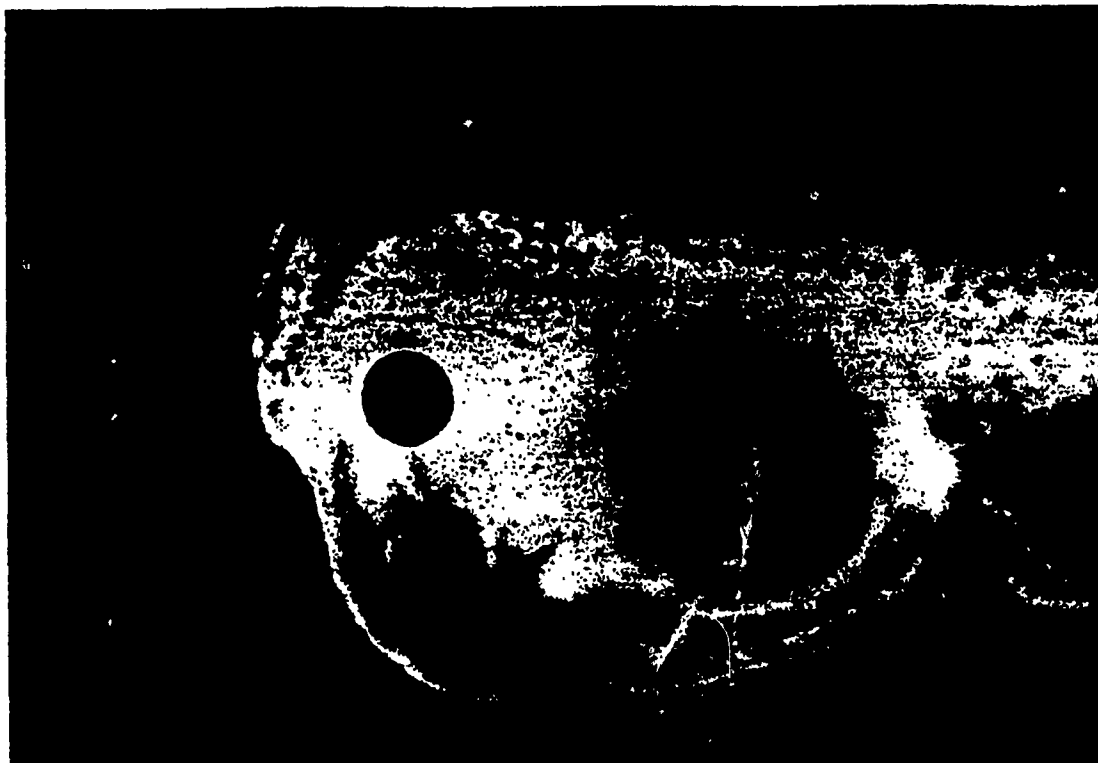


Plate 4A. Effects of a Medium Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.3 mg/ml 5-Azacytidine.



Plate 4B. Effects of a High Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.8 mg/ml 5-Azacytidine.

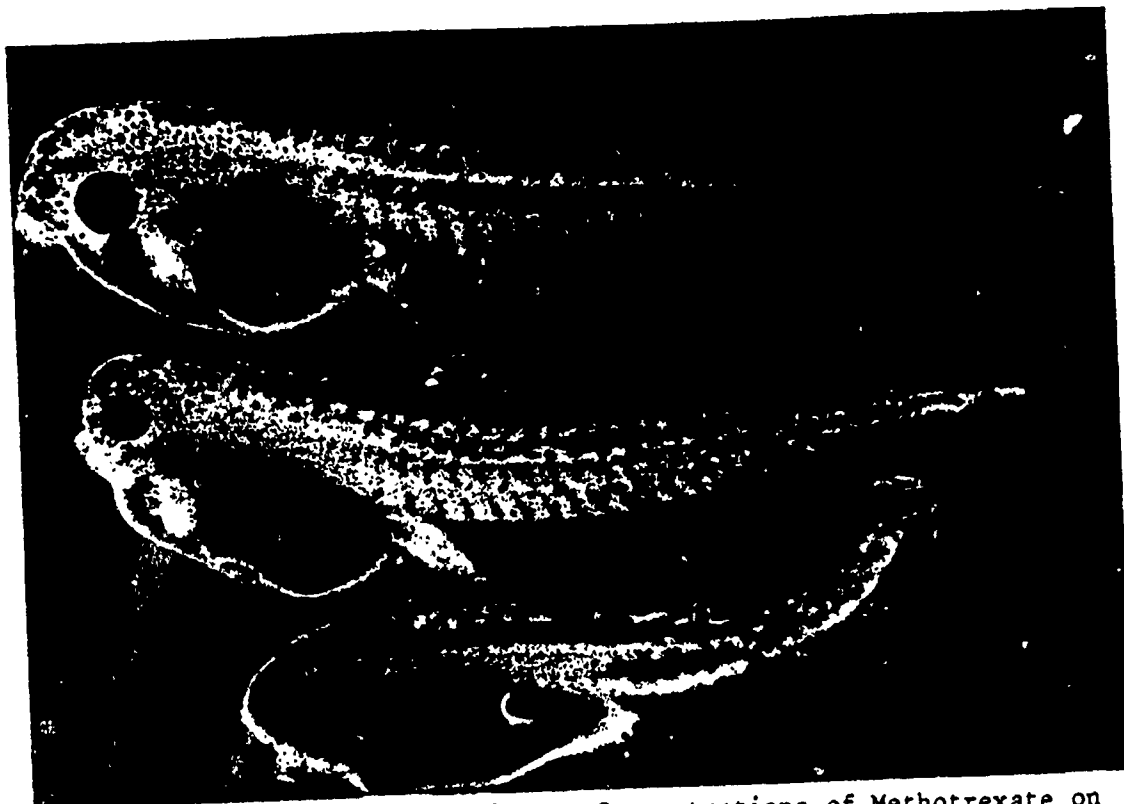


Plate 5A. Effects of Different Concentrations of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Refer to Plate 4A for control embryo. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: 0.01 mg/ml, 0.05 mg/ml, 0.5 mg/ml.

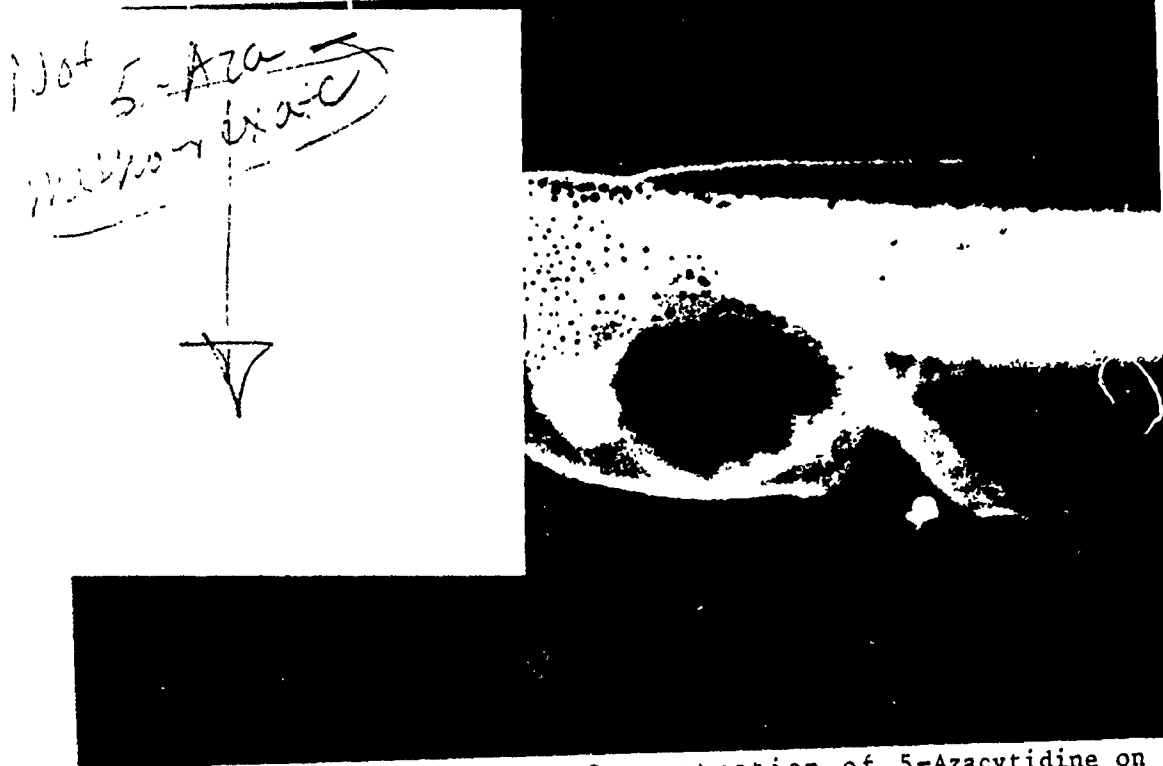


Plate 5B. Effects of a Low Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.01 mg/ml Methotrexate.

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Methotrexate: Methotrexate is a non-variable positive teratogen that acts as a folic acid antagonist and a nucleic acid synthesis inhibitor. It is listed on the Smith list (22) as a positive in both human and animal studies. This assessment is confirmed by all of the citations listed in Shepard (34). Sabourin and Faulk (14) obtained a TI between 1.6-2.5 in FETAX while Dumont obtained a TI of 5.94. We obtained a TI of 43 in the first range test and 23 in the first definitive. We just completed a second definitive test on Methotrexate this week and obtained a TI of 17 with excellent dose-response data. These values are higher than that of Dumont who reported a TI 5.94 for methotrexate. Figures 19 and 21 show the mortality and malformation dose-response curves for Methotrexate. The curve for Fig 19 is a range finder so the points on the curve are few in number and widely separated. The indication is clear from both Figures that there is a wide separation between the two curves. This separation is borne out by the high TI value and suggests that Methotrexate is a strong teratogen.

Growth inhibition by Methotrexate is shown in Figs. 20 and 22. As in the case of 5-Azacytidine, there is a significant growth reduction at concentrations that are less than 20% of the 96-hr LC50, a sharp slope in the curve and a greater than 20% decrease in body length at the highest concentrations. All of these characteristics are good indicators of a strong developmental toxicant that can inhibit growth.

Plate 6A and B shows the effect of both medium and high concentrations of Methotrexate on Xenopus development at 96 hr. As is true for most inhibitors of nucleic acid synthesis, most of the organs of the body are affected. The eyes do not suffer great effects but reduced head size is apparent along with facial malformations. Cardiac malformations are common as is miscoiling of the gut. Plate 6B shows are very abnormal looking embryo exhibiting an upward kinked tail and disrupted myomeres.

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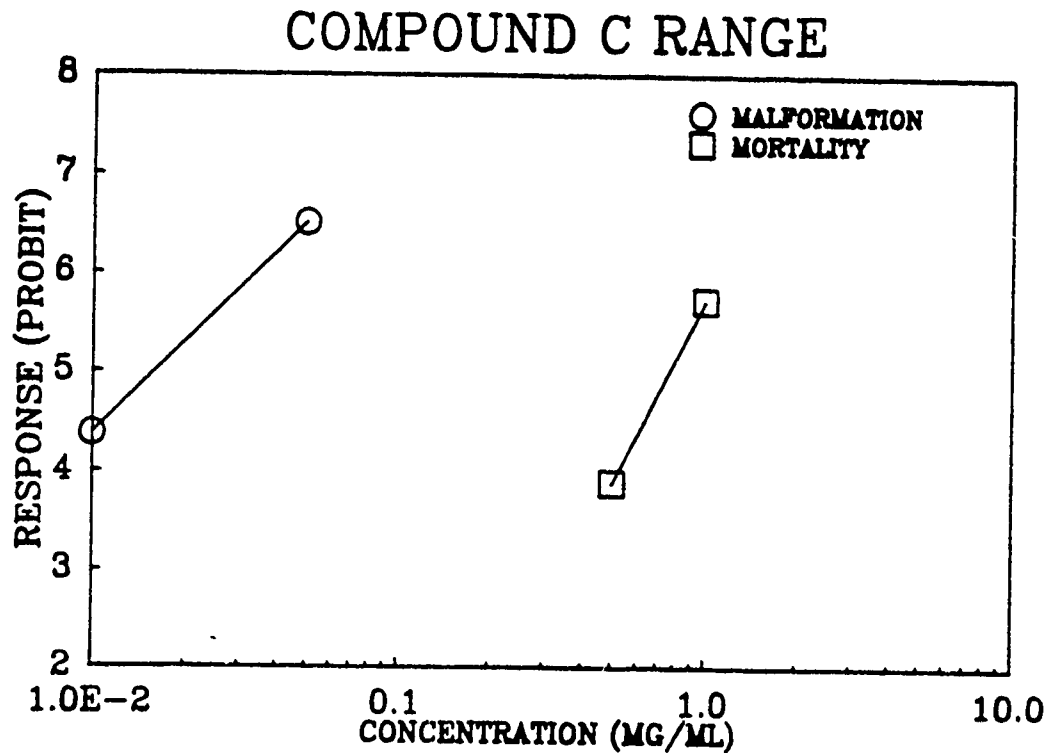


Figure 19. 96-h Mortality and Malformation Dose-Response Curves for Methotrexate Range Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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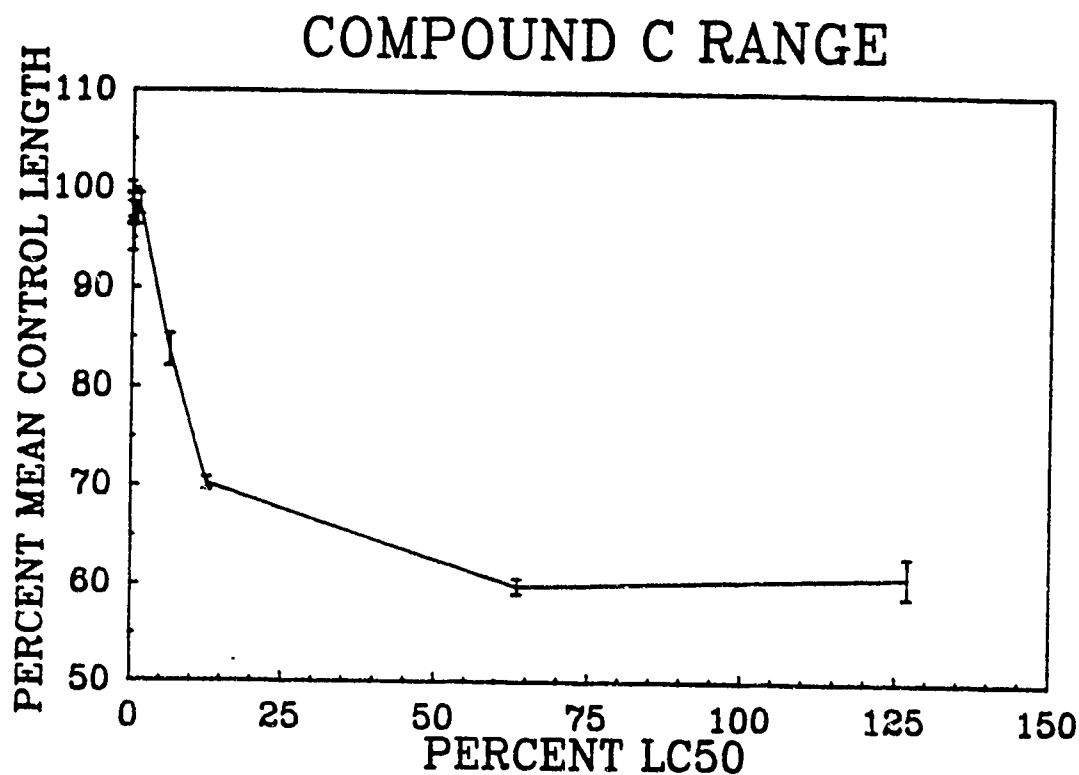


Figure 20. 96-h Growth Dose-Response Curve for Methotrexate Range Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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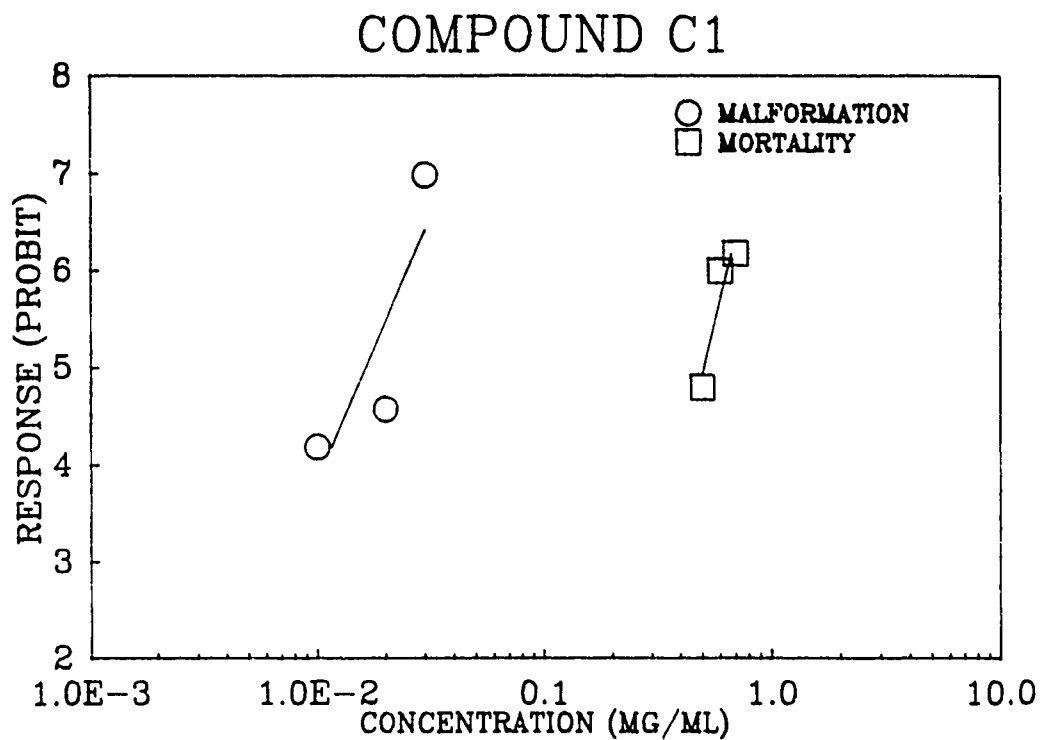


Figure 21. 96-h Mortality and Malformation Dose-Response Curves for Methotrexate Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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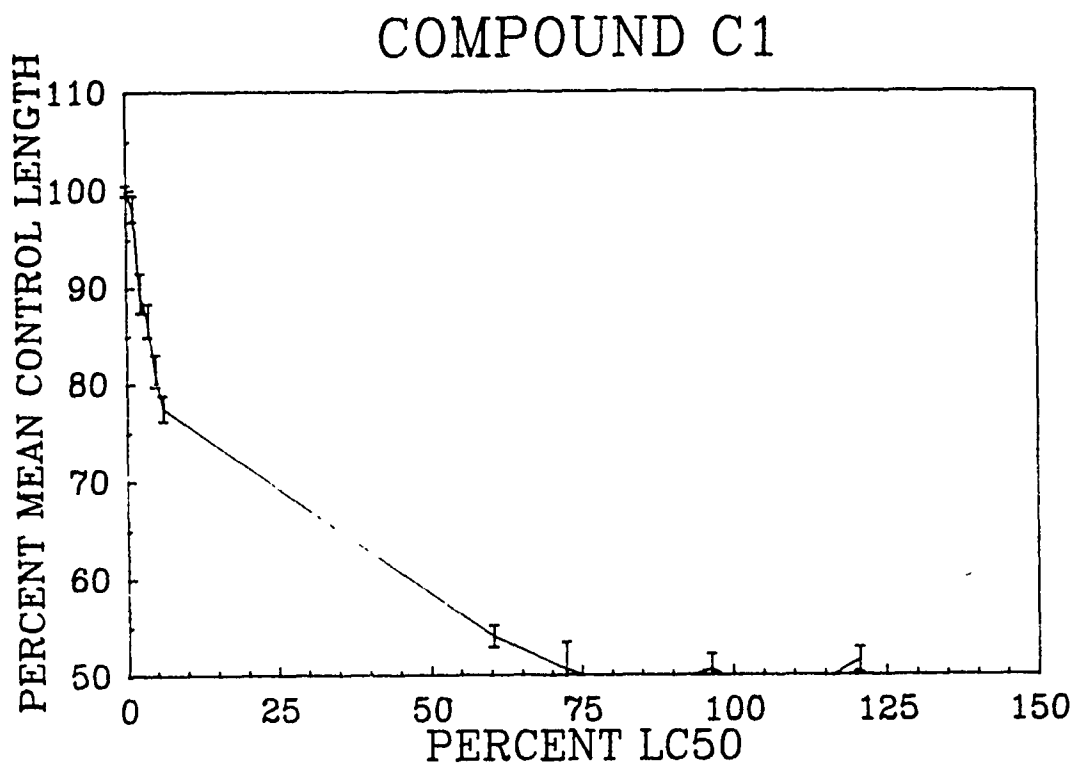


Figure 22. 96-h Growth Dose-Response Curve for Methotrexate, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

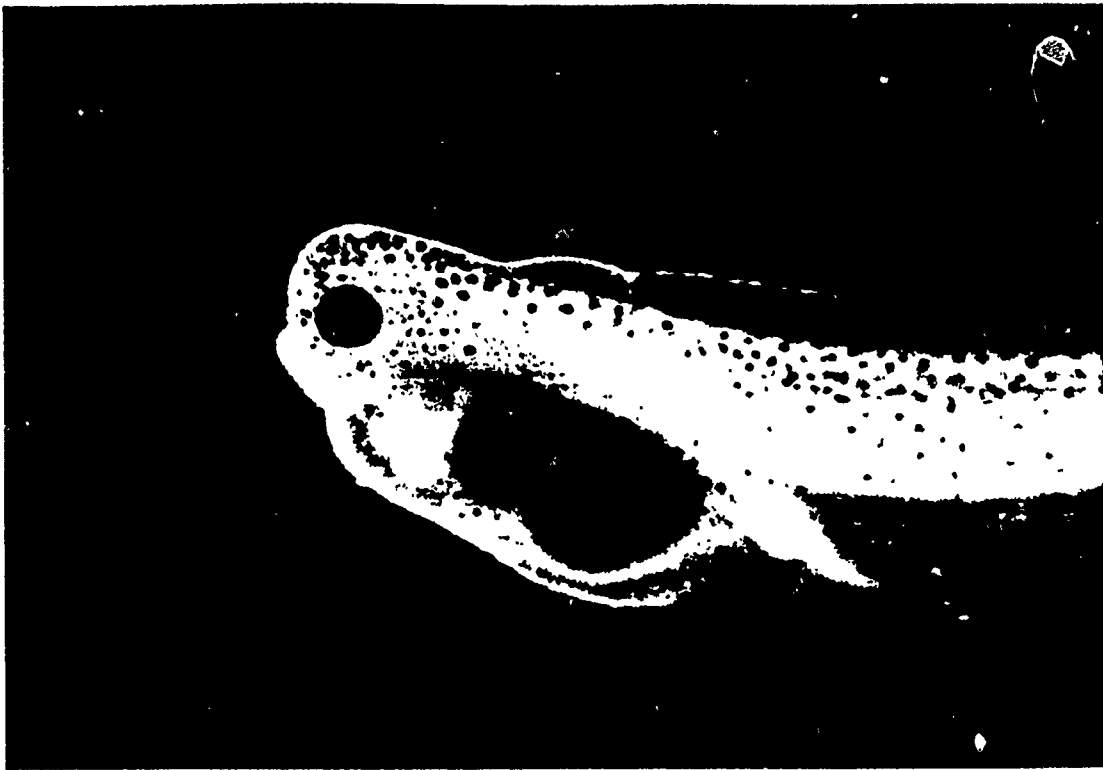


Plate 6A. Effects of a Medium Concentration of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.05 mg/ml Methotrexate.



Plate 6B. Effects of a High Concentration of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.5 mg/ml Methotrexate.

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d-Pseudoephedrine: The reports listed in Shepard (34) suggested that d-Pseudoephedrine was a negative. While Epinephrine, a related compound, was a developmental toxicant, Ephedrine, a compound more closely related to d-Pseudoephedrine, did not share this developmental toxicity. Table 1 shows that without metabolic activation (first listing) the mean TI for d-Pseudoephedrine was 1.8. We consider a compound having a TI value over 1.5 to have developmental toxicity and all three TI values listed in Table 1 are above 1.5. Additionally, the 96-hr LC and EC50 values are very close to one another indicating that we were able to precisely repeat these experiments. The three mortality-malformation curves (Figs. 23, 25, and 27) for d-Pseudoephedrine clearly show separation between them. The excellent fit of the data points indicated that the regression lines accurately reflected the dose-response curve. When we obtain results that were not anticipated, we utilize our in vitro metabolic activation system employing rat liver microsomes. The second listing for d-Pseudoephedrine shows the results from a limited series of experiments. Without metabolic activation, we essentially repeated the results of the first three experiments. The number of data points did not allow us to confidently predict the TI. With metabolic activation all of the mortality and malformation was eliminated following d-Pseudoephedrine exposure. We interpret this data to mean that d-Pseudoephedrine would not pose any particular problems in in vivo mammalian experiments.

Figures 24, 26 and 28 show the grow inhibition data for d-Pseudoephedrine. The shape of the curve is consistent for either nonteratogen or a weak teratogen. Over the first 20% of the 96-hr LC50 the growth inhibition is much less than 20%. The slope is not too steep compared to Figure 22 for Methotrexate. The overall maximum reduction in growth for d-Pseudoephedrine is about 30-35%. This is more typical of a weak teratogen than a nonteratogen. All three growth inhibition curves are nearly superimposable indicating good precision in the data.

Plates 7 and 8 show the effects of different d-Pseudoephedrine concentrations on Xenopus development after 96 hr of exposure. Plate 7A shows typical embryos from three separate concentrations of d-Pseudoephedrine and a control embryo (top). The embryo from the 0.25 mg/ml concentration shows the effects seen at about the 96 hr EC50 (malformation) (Plate 7b). This embryo exhibits moderate malformations particularly in the head and face regions and this was typical of most of the malformed embryos. Other typical malformations in the concentration range were loose gut coiling and cardiac edema. Many embryos showed blisters as well. In higher concentrations these same abnormalities still dominated except that they were more severe. Plates 8A and 8B show the more severe malformations that occur at higher concentrations. Note that 8A shows an embryo that exhibits failure of the choroid fissure of the eye to fuse at the ventral aspect. Ocular and cardiac edema are apparent. Plate 8B shows a ventral view of an embryo near the 96-hr LC50. The gut is distended and not well coiled. Ocular edema is obvious and there are blisters near the anus.

In summary, d-Pseudoephedrine is a weak to moderate teratogen unless there is metabolic breakdown present. It then rapidly loses much of its embryotoxicity and teratogenicity. Without the metabolic activation system d-Pseudoephedrine would have been scored as a false positive in FETAX.

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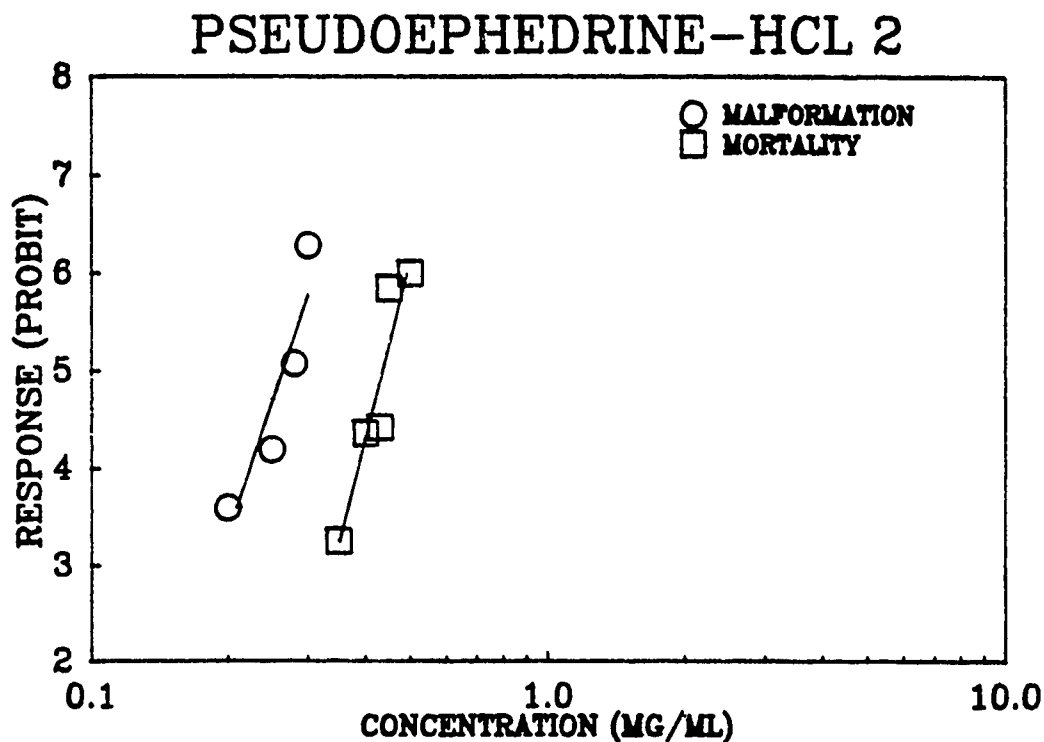


Figure 23. 96-h Mortality and Malformation Dose-Response Curves for Psuedoephedrine Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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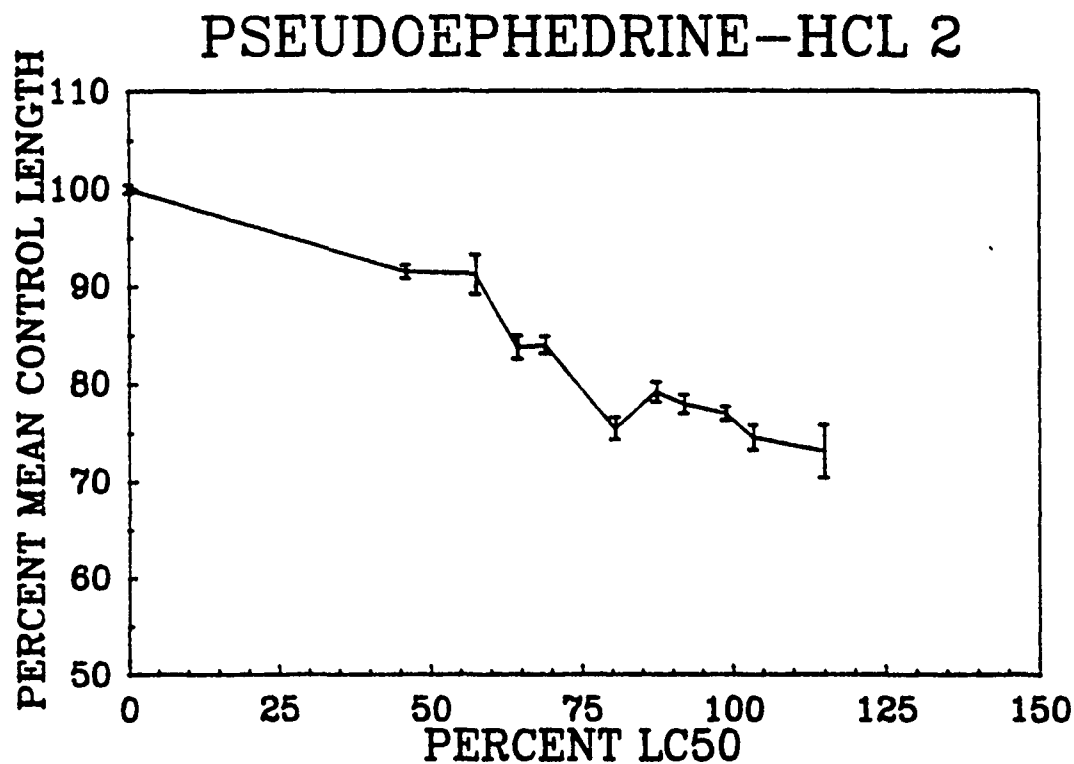


Figure 24. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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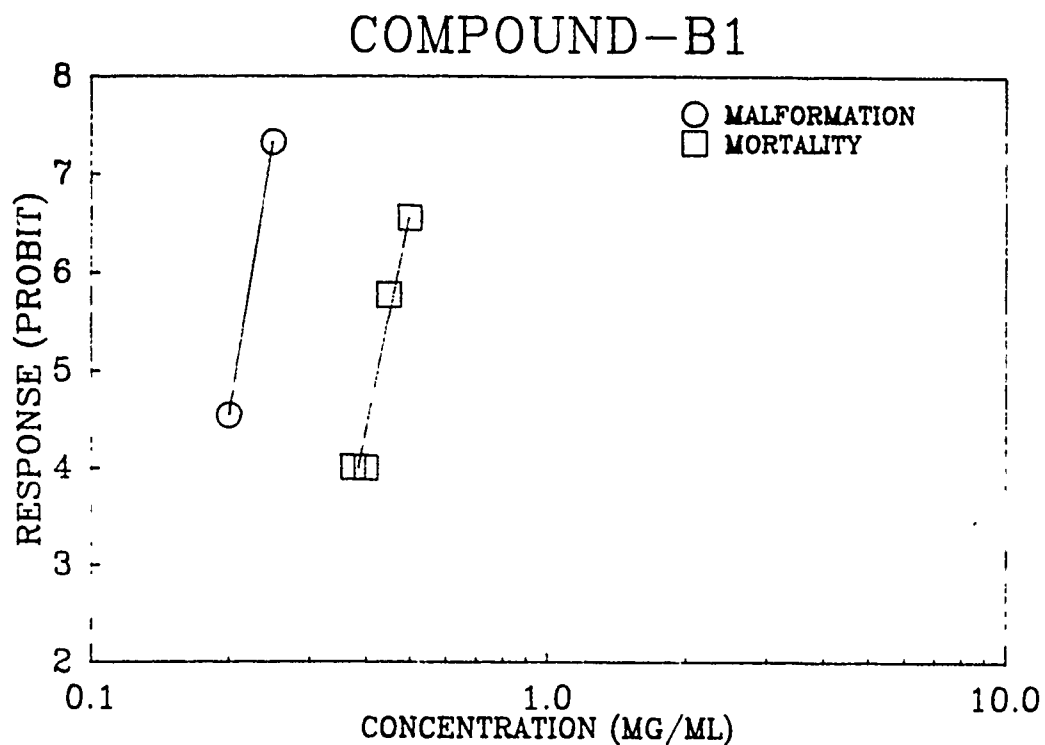


Figure 25. 96-h Mortality and Malformation Dose-Response Curves for Psuedoephedrine Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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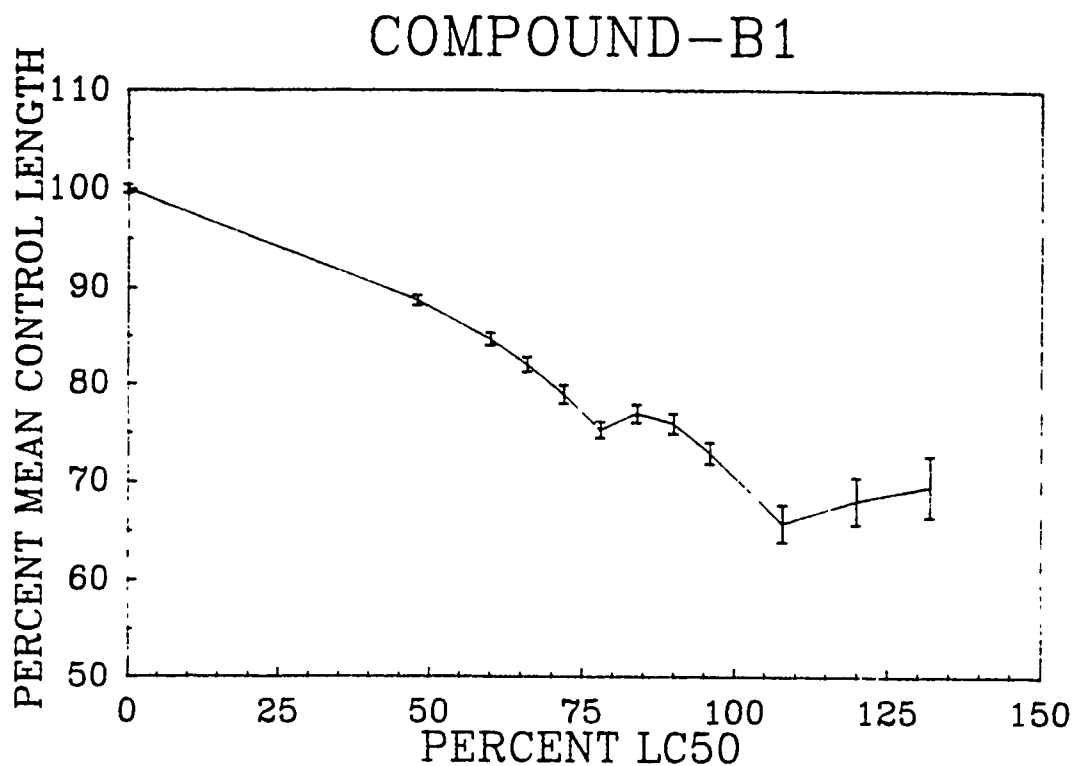


Figure 26. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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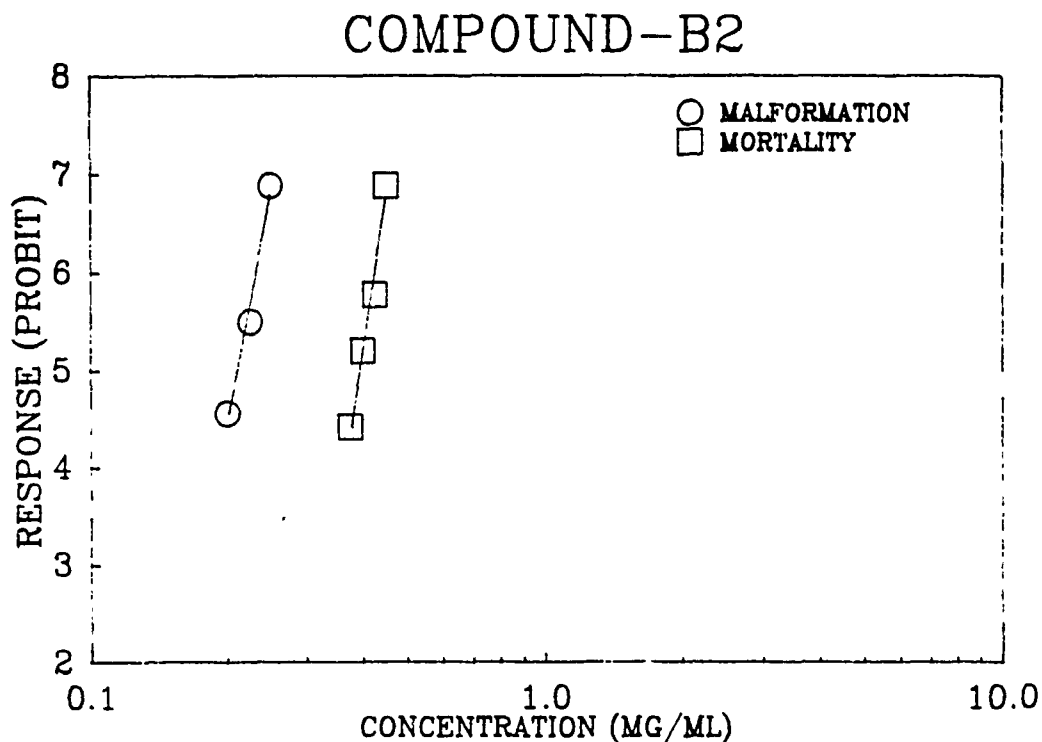


Figure 27. 96-h Mortality and Malformation Dose-Response Curves for Psuedoephedrine Definitive Test #3. . The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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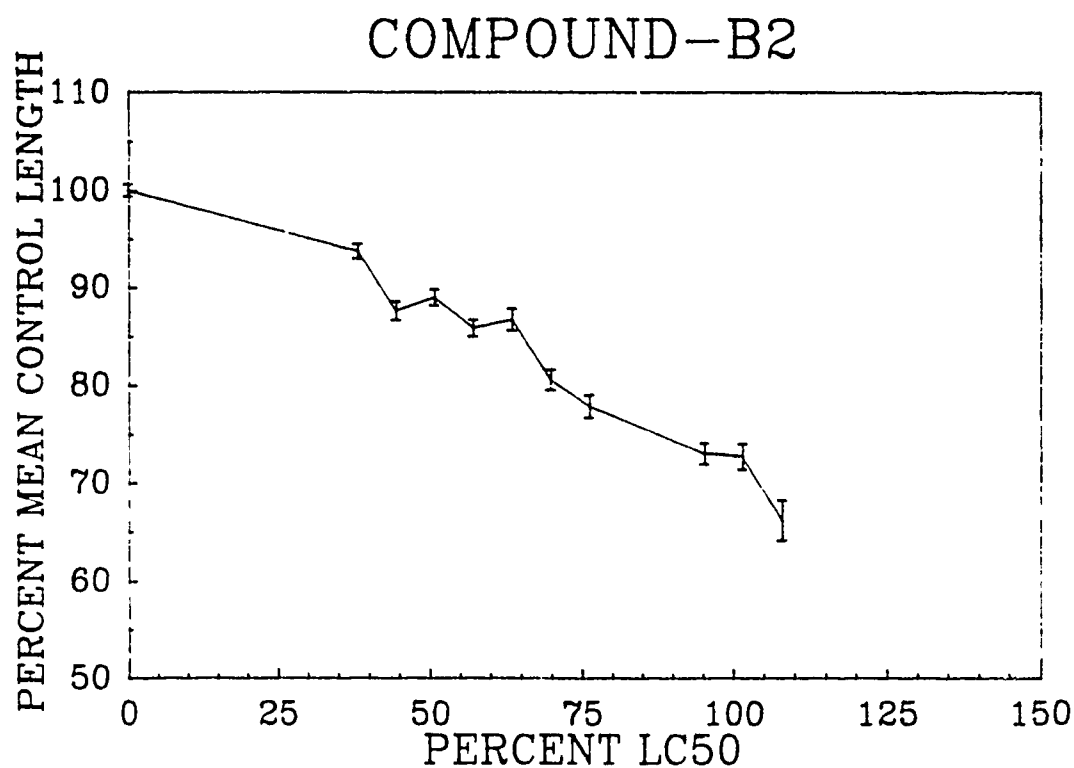


Figure 28. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #3. . The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 7A. Effects of Different Concentrations of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.25 mg/ml, 0.3 mg/ml, 0.5 mg/ml.

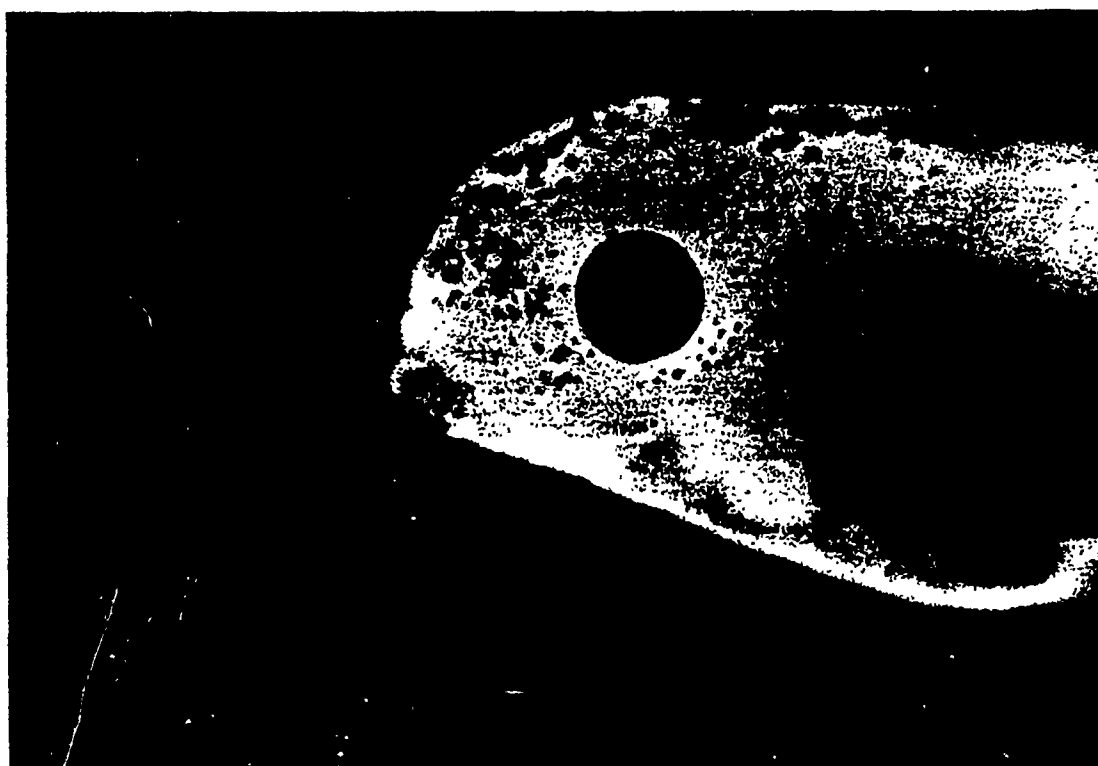


Plate 7B. Effects of a Low Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.25 mg/ml Pseudoephedrine.



Plate 8A. Effects of a Medium Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.3 mg/ml Pseudoephedrine.



Plate 8B. Effects of a High Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on brain and eye region. Embryo exposed to 0.5 mg/ml Pseudoephedrine.

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B. Phase II

ResultsValidation

The developmental toxicity of five compounds was evaluated with the Frog Embryo Teratogenesis Assay: *xenopus* (FETAX) and the results were compared to mammalian literature. Small cell *xenopus laevis* blastulae were exposed to ascorbic acid, sodium selenate, coumarin, serotonin and 13-cis retinoic acid for 96 hr. Three separate static-renewal assays were conducted for each compound. Teratogenic potential of the test materials was determined based on Teratogenic Index values [$TI = LC50/EC50$ (malformation)], types and severity of induced malformations and embryo growth. Ascorbic acid had little or no teratogenic potential. Sodium selenate and coumarin tested as having moderately positive teratogenic potential. Serotonin scored as having moderately strong teratogenic potential and 13-cis retinoic acid scored as having strong teratogenic potential. Results were consistent with mammalian data and support the use of FETAX for the screening of developmental toxicants.

At least one range and three definitive concentration-response tests were conducted for each compound. Tests run separately by different technicians were analyzed together statistically. The pH of all compounds tested was between 7.0 and 8.0. Throughout the test embryos were cultured at $24^{\circ}C \pm 1$.

Final results from the definitive tests with FETAX are presented in Table 4.

Representative concentration-response and growth-inhibition curves for the five compounds are illustrated in Figures 1 and 2, respectively.

In this investigation the FETAX solution control mortality and malformation rates were 44 of 1300 (3.4%) and 72 of the 1256 survivors (5.7%), respectively. Control data for dimethyl sulfoxide, the solvent used in the testing of coumarin, was 1 of 130 (0.8%) for mortality and 5 of 129 survivors (3.8%) for malformation. Acceptable rates of control mortality and malformation are generally $< 10\%$.

FETAX determines teratogenic potential by comparing TI values, embryo growth, and the type and severity of induced malformations. In general, TI values < 1.5 indicate low teratogenic potential and higher values indicate an increase in the potential hazard.^{2,4,8-12} With higher TI values, the mortality and malformation dose-response curves become separated and the potential for the production of deformed embryos in the absence of lethality increases³.

In this investigation ascorbic acid exhibited an overlapping of the curves as presented in Figure 29. It is, therefore, considered embryo-lethal at high concentrations and is not a potential teratogen. Sodium selenate and coumarin represent compounds with increasing separation of the curves and

TABLE 4.
Developmental Toxicity of Five Compounds Tested with FETAX

| <u>Compound</u> | <u>Test#</u> | <u>LC50^a</u> | <u>EC50^a</u> | <u>TI</u> | <u>MCIG^b</u> | <u>MCIG^c</u> |
|---------------------------------------|--------------|--|---|-----------|-------------------------|-------------------------|
| Ascorbic acid CAS 50-81-7 | 1 | 19.2 (17.8-20.7) | 11.6 (10.2-13.2) | 1.7 | 10.0 | 52 |
| | 2 | 20.3 (18.7-21.9) | 12.8 (12.4-13.3) | 1.6 | 10.0 | 49 |
| | 3 | 19.6 (18.9-20.3) | 12.0 (10.4-13.8) | 1.6 | 10.0 | 51 |
| Sodium selenate CAS 13410-10-0 | 1 | 0.017 (0.016-0.017) | 0.006 (0.002-0.013) | 3.0 | 0.014 | 82 |
| | 2 | 0.019 (0.017-0.019) | 0.007 (0.006-0.008) | 2.8 | 0.006 | 32 |
| | 3 | 0.027 (0.026-0.029) | 0.009 (0.006-0.012) | 3.1 | 0.008 | 30 |
| Coumarin CAS 91-64-5 | 1 | 0.15 (0.14-0.15) | 0.038 (0.025-0.059) | 4.0 | 0.01 | 7 |
| | 2 | 0.14 (0.13-0.14) | 0.038 NA | 3.5 | 0.05 | 36 |
| | 3 | 0.10 NA | 0.045 (0.037-0.055) | 2.2 | 0.04 | 40 |
| Serotonin CAS 153-98-0 | 1 | 2.74 (2.55-2.93) | 0.35 (0.19-0.66) | 7.8 | 0.25 | 9 |
| | 2 | 3.27 (3.18-3.36) | 0.39 (0.21-0.72) | 8.4 | 0.60 | 18 |
| | 3 | 3.21 NA | 0.48 (0.43-0.54) | 6.7 | 1.00 | 3 |
| 13-cis Retinoic acid CAS 4759-48-2 | 1 | 37x10 ⁻⁶ (25-57x10 ⁻⁶) | 3x10 ⁻⁶ (2-3x10 ⁻⁶) | 18.8 | 7x10 ⁻⁶ | 19 |
| | 2 | 18x10 ⁻⁶ (16-22x10 ⁻⁶) | 2x10 ⁻⁶ (1-3x10 ⁻⁶) | 9.2 | 20x10 ⁻⁶ | NA |
| | 3 | 36x10 ⁻⁶ (34-37x10 ⁻⁶) | 4x10 ⁻⁶ (3-4x10 ⁻⁶) | 10.1 | 10x10 ⁻⁶ | 28 |

a mg/ml with (95% confidence interval).

b Minimum Concentration to Inhibit Growth as mg/L.

c Minimum Concentration to Inhibit Growth as a percent of LC50.

NA Not available.

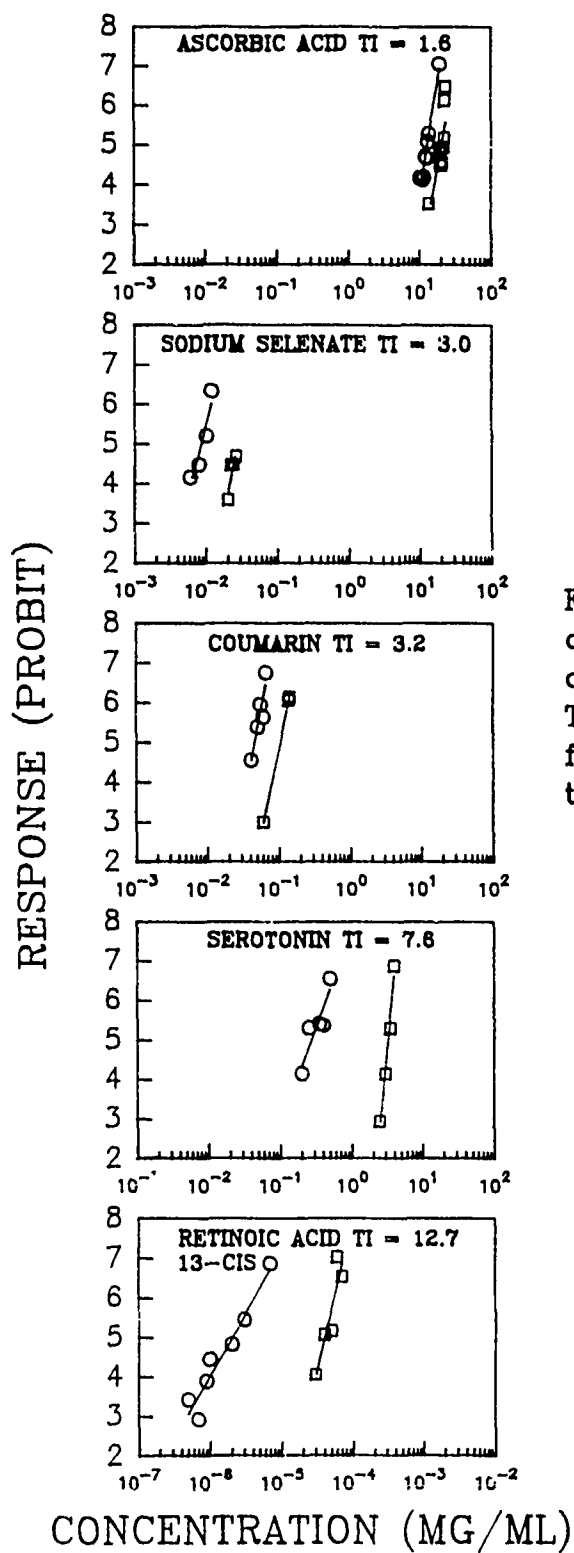


Figure 29. Representative concentration-response curves and respective Teratogenic Index values for the five compounds tested with FETAX.

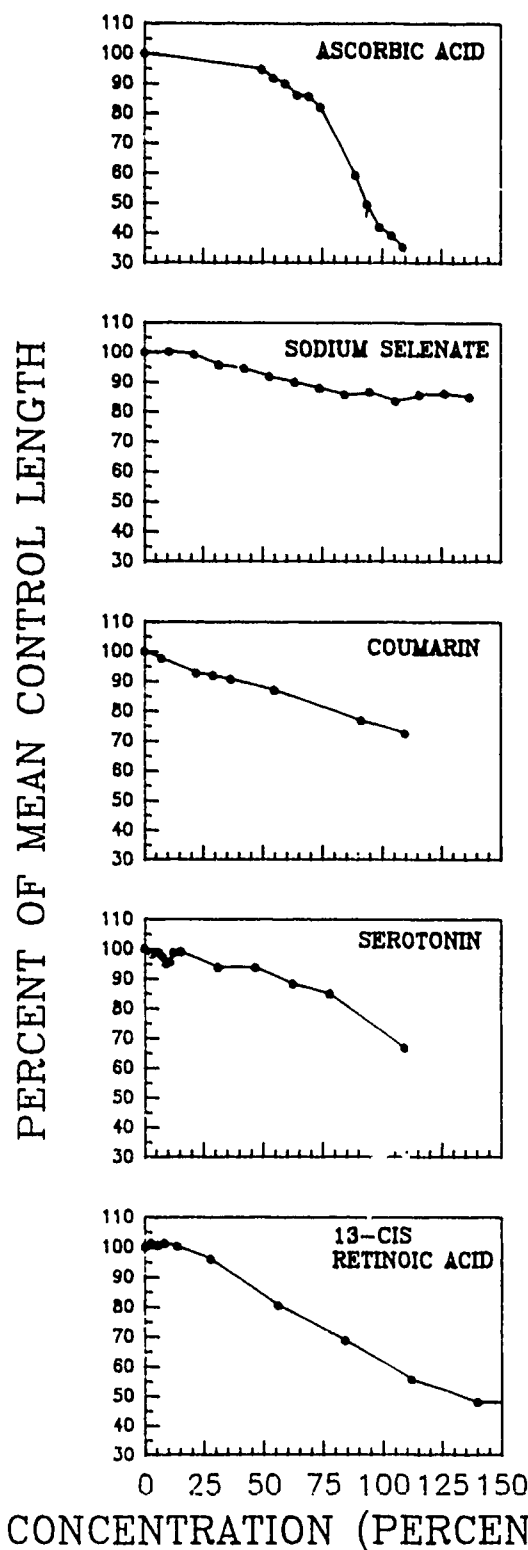


FIGURE 30 Representative embryo growth curves for the five compounds. Concentrations are expressed as percent of the respective compound LC50. Growth is expressed as percent of mean FETAX solution control length.

potential teratogenic hazard. Serotonin and 13-cis retinoic acid exhibit even wider separation of the curves, and are examples of compounds with strong teratogenic hazard. Because all chemicals are potential teratogens if administered in appropriate doses at sensitive stages of development¹⁷, it is important to consider the types and severity of terata and the concentrations at which they occur. Compounds with TI values < 1.5 may pose a hazard to developing organisms, possibly as embryotoxins.

Developmental toxicity may also be assessed by considering the MCIG (expressed as % compound LC50)^{2-4,10}. Rates of growth inhibition (i.e. slope) and overall reduction in embryo growth vary with the severity of the teratogen. Dawson et al.⁴ suggest that compounds with significant teratogenic potential generally inhibit growth at concentrations $< 30\%$ of the respective LC50 values. Ascorbic acid begins to inhibit growth at $> 50\%$ of the LC50. Sodium selenate and coumarin cause inhibition between 28 and 48% of the LC50. Serotonin and 13-cis retinoic acid cause inhibition between 10% and 16% of each particular LC50. In addition, serotonin and 13-cis retinoic acid with the highest TI values show a sharper decrease in slope compared to the other compounds. This characteristic is presented in Figure 30 by the growth-inhibition curves.

The five compounds presented here have been selected as part of the validation process of FETAX because of the availability of mammalian literature for each¹⁸⁻¹⁹. Although FETAX results cannot be directly extrapolated to mammalian developmental toxicity tests, comparisons are beneficial in order for FETAX to be useful as a screening assay.

The results of this study indicate that all five compounds agree with the majority of mammalian literature available regarding teratogenicity. Ascorbic acid, cited as a negative teratogen in mammalian literature²⁰⁻²², tested negative in FETAX. Sodium selenate and coumarin, variable positives in mammalian literature^{23-31,32-38}, tested positive in FETAX. Serotonin and 13-cis retinoic acid, listed as positives in mammalian literature^{39-41,42-44}, tested positive in FETAX. Overall, FETAX currently has a predictive accuracy of 89% including compounds tested with metabolic activation.

1. Ascorbic acid

The most common malformation induced by ascorbic acid was failure of the gut to coil. At concentrations > 10 mg/ml loose gut coiling was common along with slight musculoskeletal kinking. At concentrations > 13 mg/ml facial, eye and brain malformations were noted. Growth was stunted and severe malformations of the gut, musculoskeletal system, face, eye and heart occurred at concentrations > 19 mg/ml.

Ascorbic acid which tested negative in FETAX, has been tested with

mice, rats and rabbits in studies following FDA Segment II guidelines. No effects were observed in rats up to 500 or 1000 mg/kg/dy²⁰⁻²¹. No effect was observed in mice up to 1000 mg/kg²¹ or in rabbits up to 500 mg/kg/dy²⁰. Frohberg et al.²¹ administered up to 1000 mg by mouth to pregnant mice and rats on days 6-15 with no adverse effects found. In fact, ascorbic acid (ascorbate) has been found to protect against the embryoletality of N-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene (NF), and decreased the number of flexure abnormalities caused by NF in a rat whole embryo culture system²².

2. Sodium Selenate

Sodium selenate at concentrations > 0.002 mg/ml resulted in embryos with edema and malformations of the gut, heart and face. Blistering was evident at concentrations > 0.012 mg/ml.

FETAX results for sodium selenate also agree with the majority of mammalian literature reports. As a potential teratogen it tested positive in FETAX. Selenium induced malformations have resulted from livestock grazing on seleniferous ranges.²³⁻²⁴ Beath et al.²⁴ reported malformations in lambs consisting of multiple cysts in eyes, microphthalmia and deformities of the extremities. Similar effects were reported with horses²⁵. However, early laboratory studies with rats and cats fed a continuous diet of selenium did not result in any malformations in the progeny²⁶.

As a result of the interest in the Kesterson Reservoir and Kesterson National Wildlife Refuge, Merced County, California, in which selenium contamination was a factor, several developmental toxicity studies have emerged. Sodium selenite and selenomethionine were tested in the laboratory with mallards and malformations found included hydrocephaly, microphthalmia, lower bill and foot defects, edema and stunted growth²⁷. High rates of embryonic mortality and abnormalities were also reported in wild aquatic bird populations at Kesterson²⁸⁻²⁹. An *in situ* study was conducted using 10 species of mammals collected from Kesterson Reservoir and a low rate of abnormalities was found.³⁰ Nobunaga et al.³¹ conducted a study with mice in which the malformation rate of sodium selenite was not significantly different from controls. Species differences in developmental toxicity caused by selenium are apparent, however FETAX results agree with the species tested other than rats and mice and further studies are needed.

3. Coumarin

Dimethyl sulfoxide (DMSO) was used as a solvent for coumarin at a concentration less than 1.1% v/v which has been found not to cause any adverse

effects in FETAX. The possibility of interactions between DMSO and coumarin causing altered rates of mortality and malformation cannot be completely discounted.

Coumarin induced musculoskeletal kinking, loose gut coiling and craniofacial malformations at concentrations > 0.01 mg/ml. Concentrations > 0.04 mg/ml induced craniofacial malformations consisting of a reduced head size and downward tilting of the head. Edema, eye and gut malformations occurred at concentrations > 0.07 mg/ml. The malformations mentioned above became more severe at concentrations > 0.13 mg/ml.

Coumarin is a compound which also has conflicting reports in the mammalian literature but is generally considered to be a developmental toxin, especially for humans. Coumarin-induced abnormalities are known as the fetal warfarin syndrome³² and the most consistent malformations in humans have been described by Shaul and Hall³³ as nasal hypoplasia, stippling of the bones, ophthalmologic abnormalities, intrauterine growth retardation and developmental delay. When administered to mice, coumarin elicited a low incidence of gross fetal malformations including cleft lip and cleft palate.³⁴ A study on rabbits and mice found that coumarin adversely affected normal implantation and placentation, but no mention was made of any malformations.³⁵ In a similar study by Hirsch et al.³⁶, rabbits exposed to coumarin gave birth to stillborn fetuses with hemorrhages. One discrepancy between nonhuman and human data may be due to the fact that in man and the baboon, coumarin is metabolized to 7-hydroxycoumarin³⁷⁻³⁸ but this metabolic pathway is relatively minor in the rat. FETAX tested coumarin as a positive and in this case was a better indicator of teratogenic hazard than some laboratory non-primate tests.

4. Serotonin

Serotonin caused minor malformations and stunting at most of the concentrations tested. Embryos gradually became smaller, shorter in length and less developed with blunter nose and looser gut coil. At concentrations > 1.0 mg/ml, microencephaly and blistering of the dorsal fin were noticed. Embryos were severely stunted at concentrations > 3.0 mg/ml.

Serotonin tested positive in FETAX and agreed with mice, rat, and human data that serotonin poses a teratogenic hazard. Defects reported in laboratory mice include kidney, abdomen, eye, limb, tail, skull, brain and CNS abnormalities.³⁹⁻⁴⁰ The teratogenic effects of serotonin in the laboratory rat include anophthalmia, hydrocephalus, exencephaly, omphalocele and vacuolization of myocardial cells.⁴¹ Reddy et al.⁴¹ reported evidence of the effects of serotonin in human pregnancy.

5. 13-Cis retinoic acid

Because of the relative insolubility of 13-cis retinoic acid, stocks were prepared by measuring 1 mg into 1 L FETAX solution, stirring and filtering with 0.45-um Millipore filter paper. The stock concentration was then determined by spectrophotometry with a wavelength setting of 354 nm and extinction coefficient of 39,800.¹⁴

13-cis retinoic acid caused loose gut coiling and musculoskeletal kinking at concentrations $> 0.5 \times 10^{-6}$ mg/ml. Concentrations $> 2.0 \times 10^{-6}$ mg/ml resulted in eye and brain malformations. Cyclopia, eye pigment ruptures, edema, spinal kinking and craniofacial abnormalities were induced in concentrations $> 10 \times 10^{-6}$ mg/ml. Plates 9-11 show typical malformations.

The teratogenicity of 13-cis retinoic acid (Vitamin A) has been observed in all species tested including rat, mouse, rabbit, monkey and also *Xenopus laevis*⁴²⁻⁴³. Nervous system and craniofacial defects are the most common terata reported. Human birth defects as a result of the use of 13-cis retinoic acid are documented and described as a syndrome of central nervous system, aural and cardioaortic defects⁴⁴. J.A.G. Geelen⁴² has published a survey on malformations reported in the literature for Vitamin A and its congeners. A strong positive result in FETAX for 13-cis retinoic acid, confirms the advice that this treatment for cystic acne should be avoided in pregnant women.

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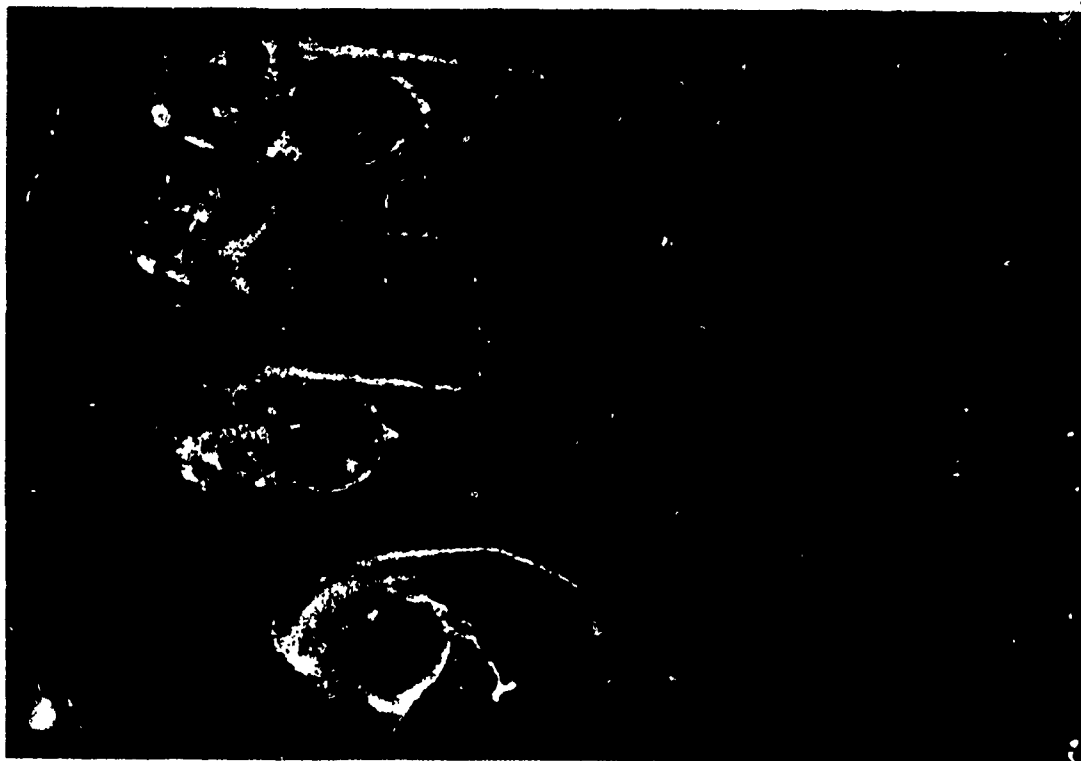


PLATE 9 The Effects of Different Concentrations of 13-cis Retinoic Acid on *Xenopus laevis*. Control embryos achieved stage 46 at the end of the 96-h exposure period. Side view presented to show effect on brain, eye, and spinal chord. From top to bottom: Control, 1.00 ng/ml, 10.0 ng/ml and 40.0 ng/ml.

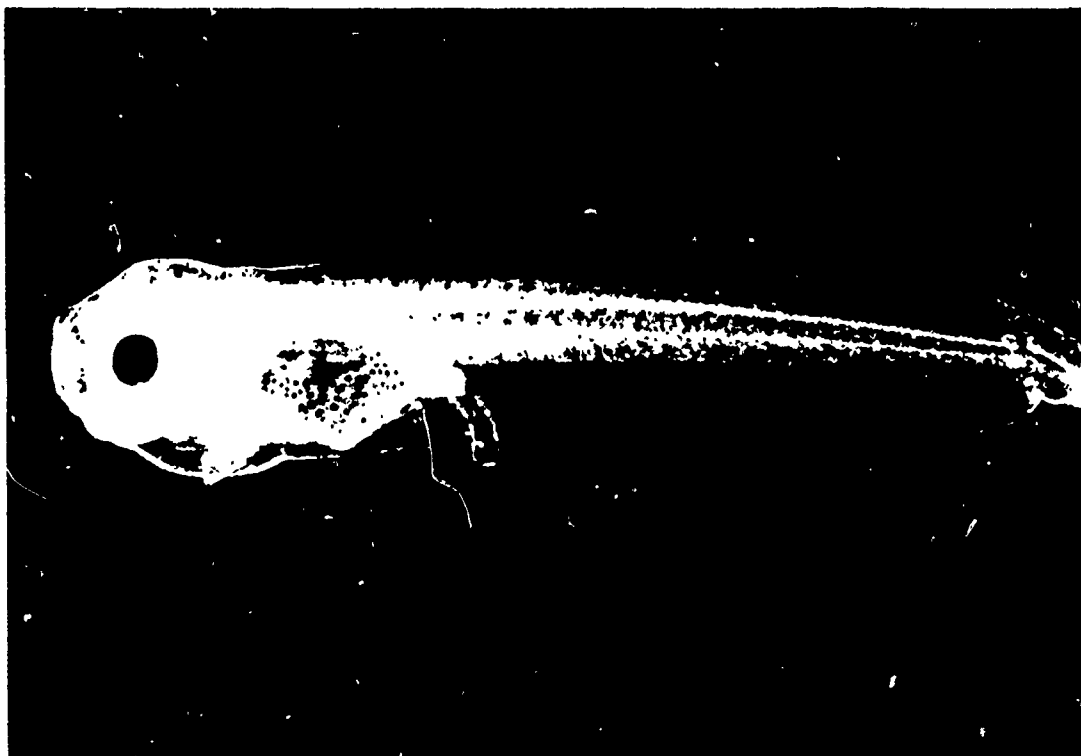


PLATE 10 Effects of 1.00 ng/ml 13-cis Retinoic Acid on *Xenopus* development. Enlarged side view presented to show cranio-facial malformation

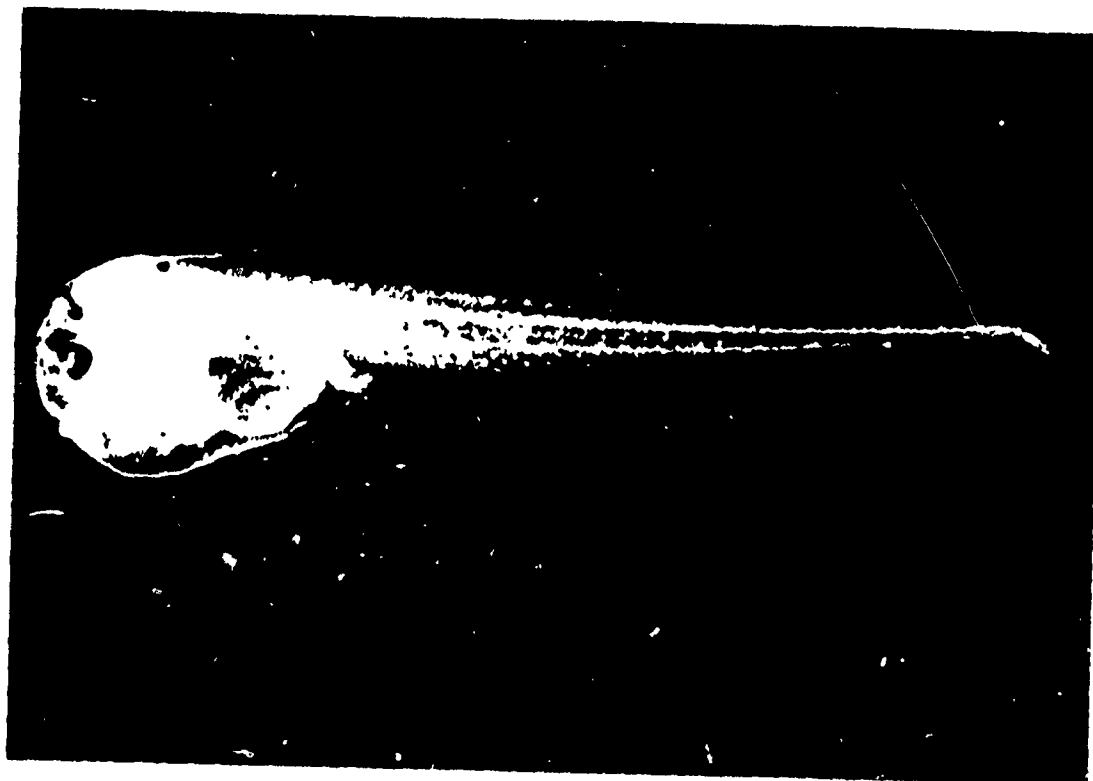


PLATE 11 Effects of 10.0 ng/ml of 13-cis Retinoic Acid. This medium concentration is presented to show the abnormal cranio-facial development that is prevalent with this compound.

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C. Phase III

Validation Study as part of the Solvent Interaction Study

Many insoluble compounds cannot presently be tested in FETAX unless it is first dissolved in a carrier solvent. While it is possible to determine the NOEC for solvents such as Triethylene Glycol, Acetone and Dimethyl Sulfoxide, there is still a possible positive or negative interaction with the test compound that could alter test results. There is ample evidence of solvent interaction in the literature. Nelson et al. (37) showed that ethanol reduced the number of neurochemical effects of 2-ethoxyethanol in rats. Demey et al. (38) found that propylene glycol when used in a IV nitroglycerin solution may cause hyperosmolality, hemolysis and lactic acidosis. Gichner and Veleminsky (39) showed that 4-12% acetone, 4-16% ethanol and 8-32% dimethylformamide potentiate the mutagenic activity of N-methyl-N'-nitro- N-nitrosoguanidine in Arabidopsis thaliana seeds. These findings make the present study imperative.

In order to conduct the solvent interaction study it was first necessary to perform dose-response curves for each of the solvents to be used as carriers and to obtain similar data for the compounds to "interact" with the solvents. From these curves the NOEC and 96-hr LC25 and EC50 (malformation) is estimated. It should be remembered that support for the latter dose-response curves is from an Oklahoma Center for the Advancement of Science and Technology grant so detailed data will not be presented here with the exception of trans-retinoic acid in order to conserve space. On the annual report, all of the data will be presented so that conclusions may be drawn.

Triethylene glycol: Triethylene glycol (TG) is very similar to other glycol derivatives but is not as toxic as them. Weyland (40) used it as a vehicle for Benzo(a)pyrene. However, Weyland does not mention any interaction with TG and Benzo(a)pyrene. TG is used as a drying agent for natural gas, in the manufacture of vinyl plasticizers and as a solvent. TG is not as toxic a solvent as DMSO or acetone in FETAX (Table 2). The mean TI of 1.12 indicates that TG is not teratogenic. The mean 96-hr LC50 is 2.45 v/v% making it much less toxic than either Acetone or DMSO (Table 2). The same is true for the 96-hr EC50(malformation) which has a mean of 2.15 v/v%. Figures 31,33 and 35 show the 96-hr mortality and malformation dose-response curves for TG. The lines are consistently superimposed on one another (or nearly so) indicating a nonteratogen. Replicability is excellent and there are many data points in the partial effects zone of 16-84% which suggests that the data is reliable. Growth inhibition curves (Figures 32,34 and 36) are repeatable and indicate that TG is a nonteratogen because there are no effects seen until concentrations are reached that are 50% of the 96-hr LC50 or greater. It is interesting that a 40% growth inhibition can be reached in surviving embryos. Plates 12 and 13 show that abnormalities are minor even at concentrations of 2% (Plate 12a & 12b). This called a low concentration in the plate legend but this refers to the other embryos exposed to higher concentrations presented on the Plate. Concentrations of 1 to 1.5% TG would be used as a carrier in FETAX as there are no observable malformations at these concentrations and growth is not affected. At higher concentrations, as shown in Plates 12 and 13, TG causes facial abnormalities and some shifting in the position of the eye. Gut and heart coiling are impaired at higher concentrations and little effect on the tail is seen. While TG proved to be the least toxic and teratogenic of the proposed carrier solvents it is not the best solvent and will not carry all test compounds into solution.

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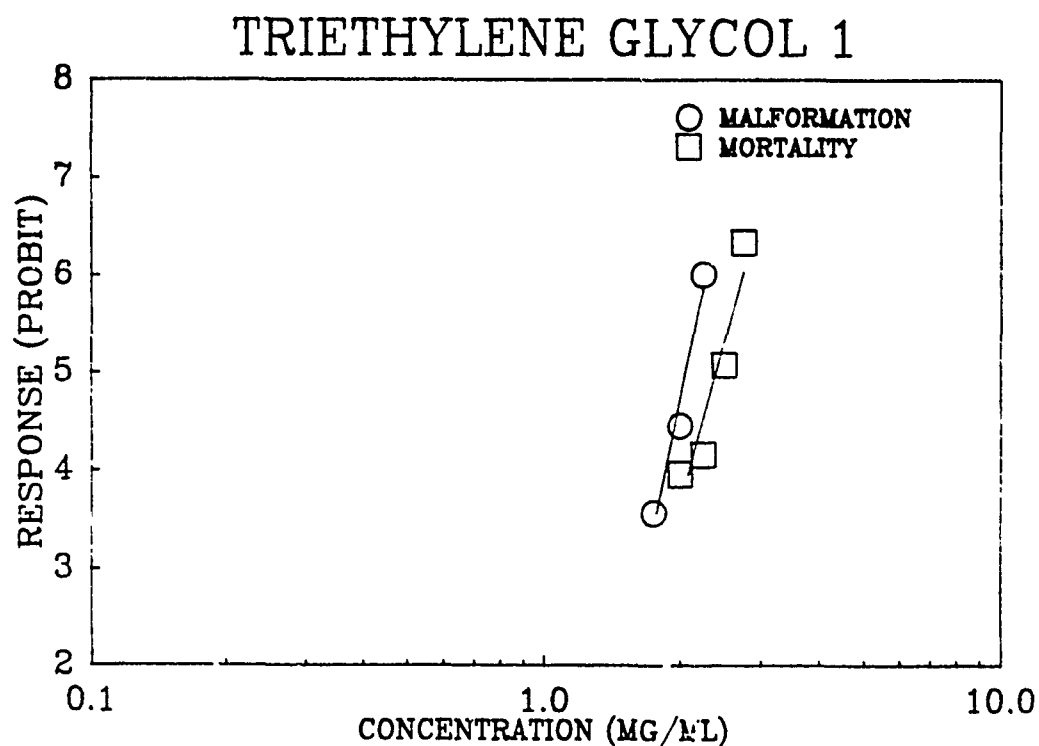


Figure 31. 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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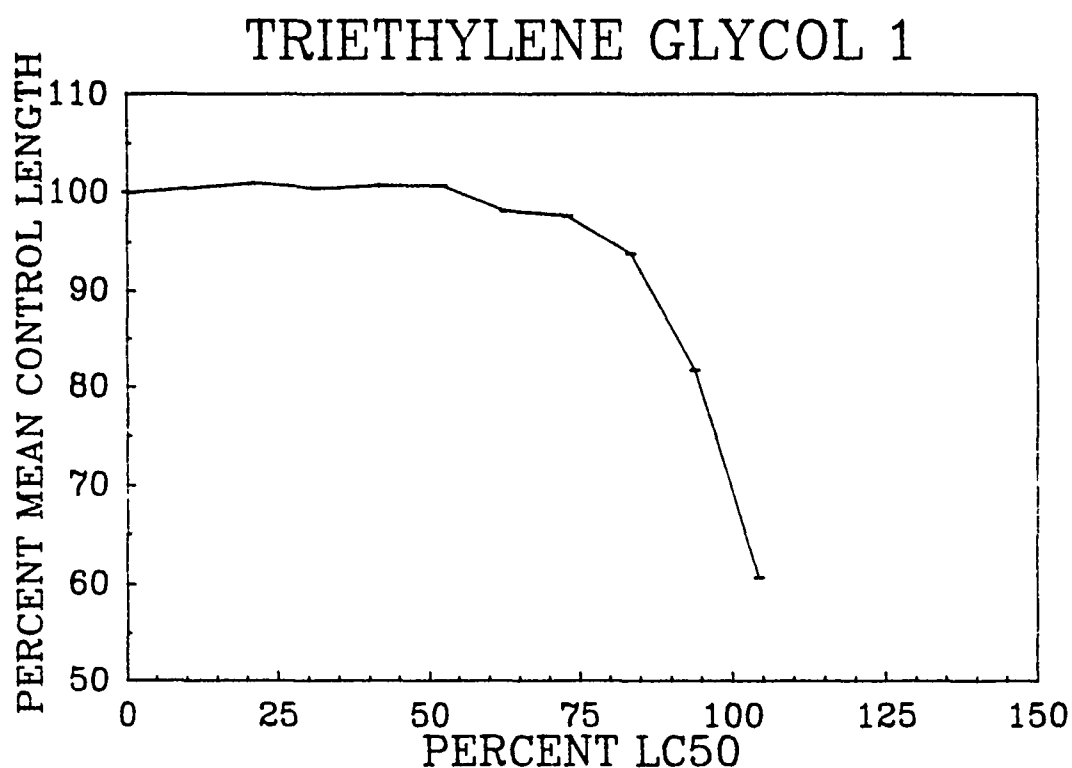


Figure 32. 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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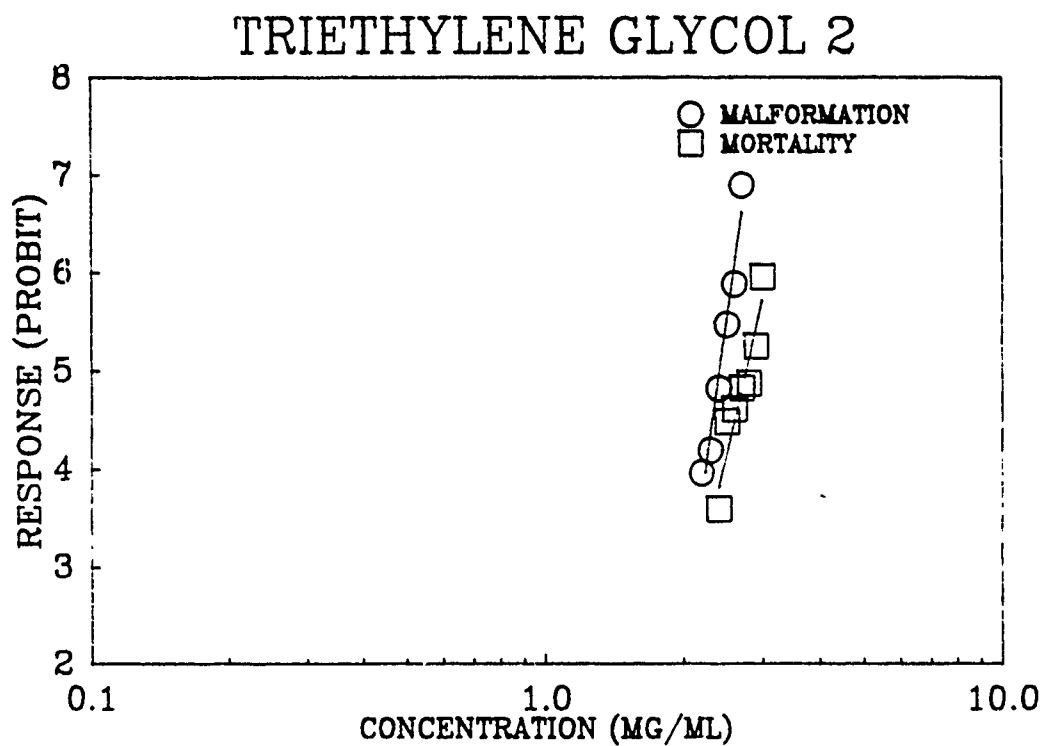


Figure 33. 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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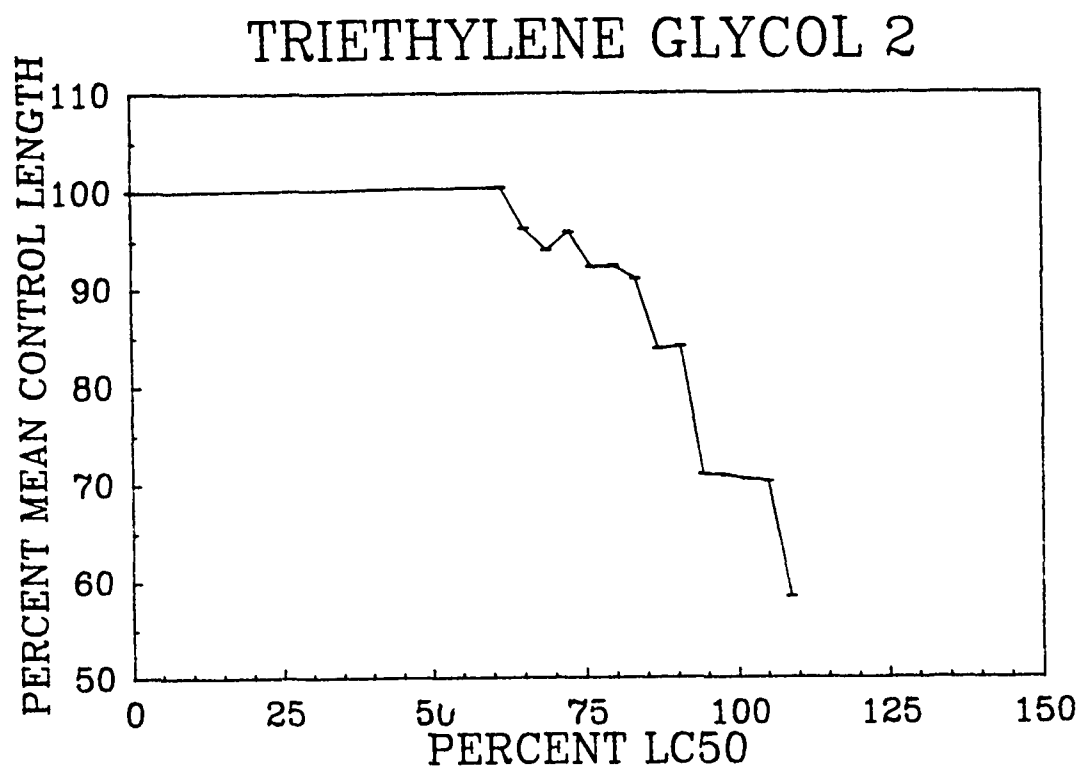


Figure 34 . 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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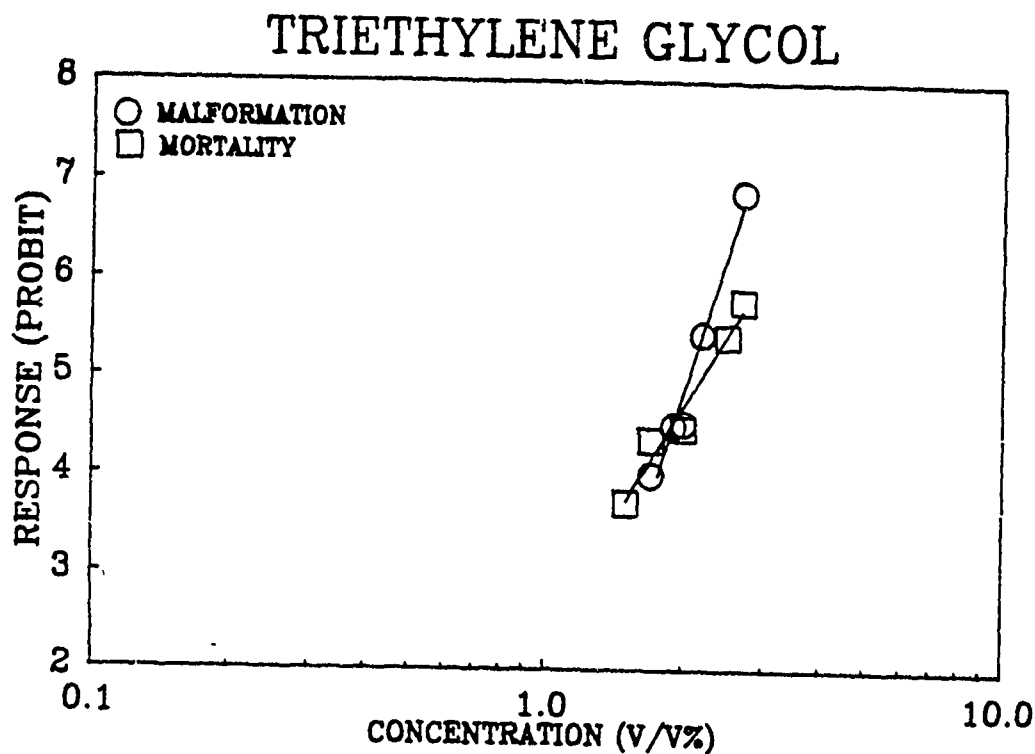


Figure 35 . 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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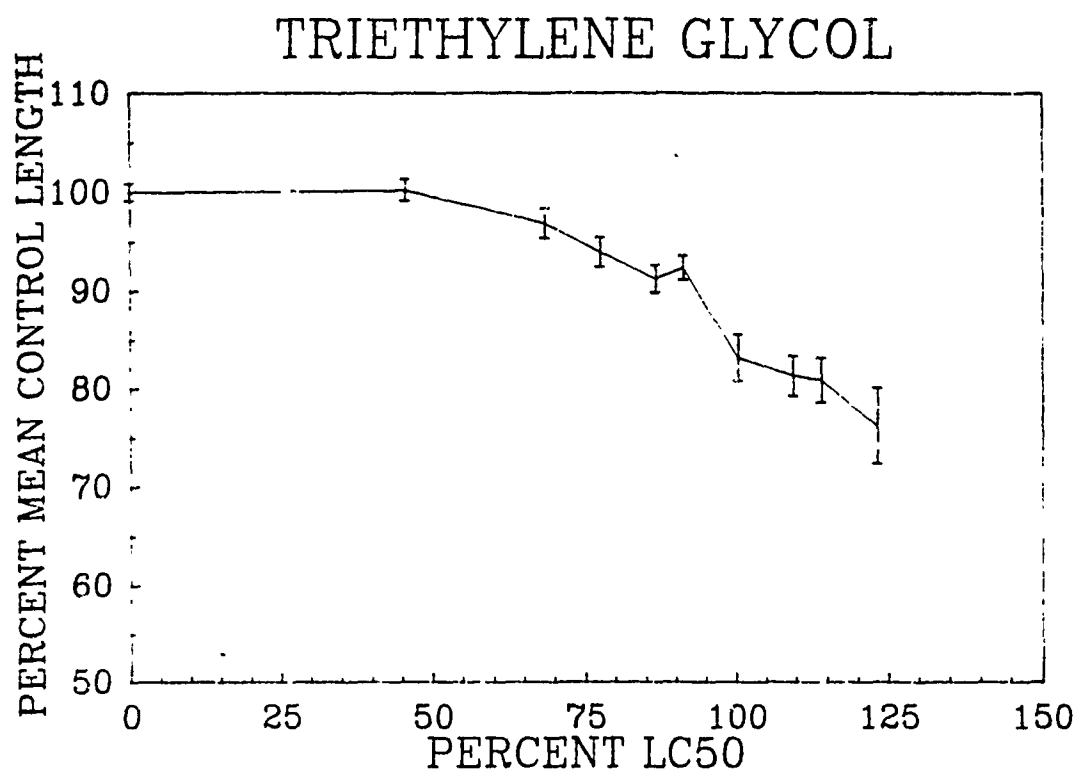


Figure 36. 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

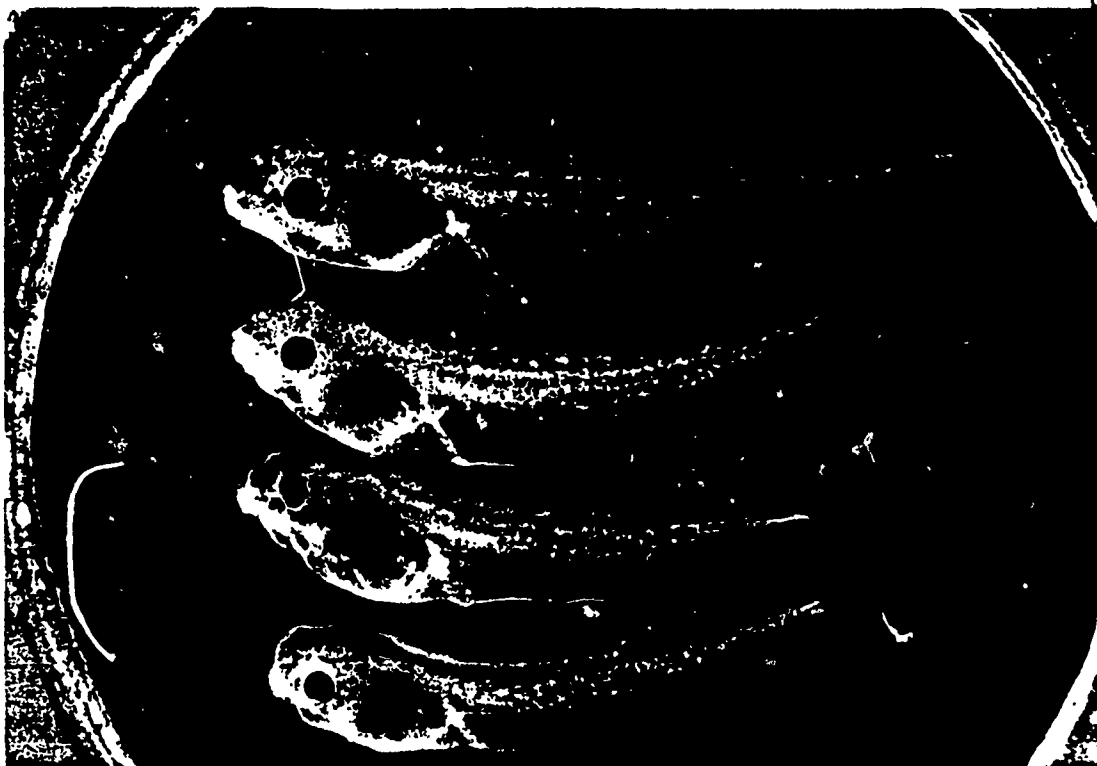


Plate 12a. Effects of Different Concentrations of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2% v/v, 2.4% v/v, 2.7% v/v.



Plate 12b. Effects of a Low Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 2% v/v Triethylene glycol.

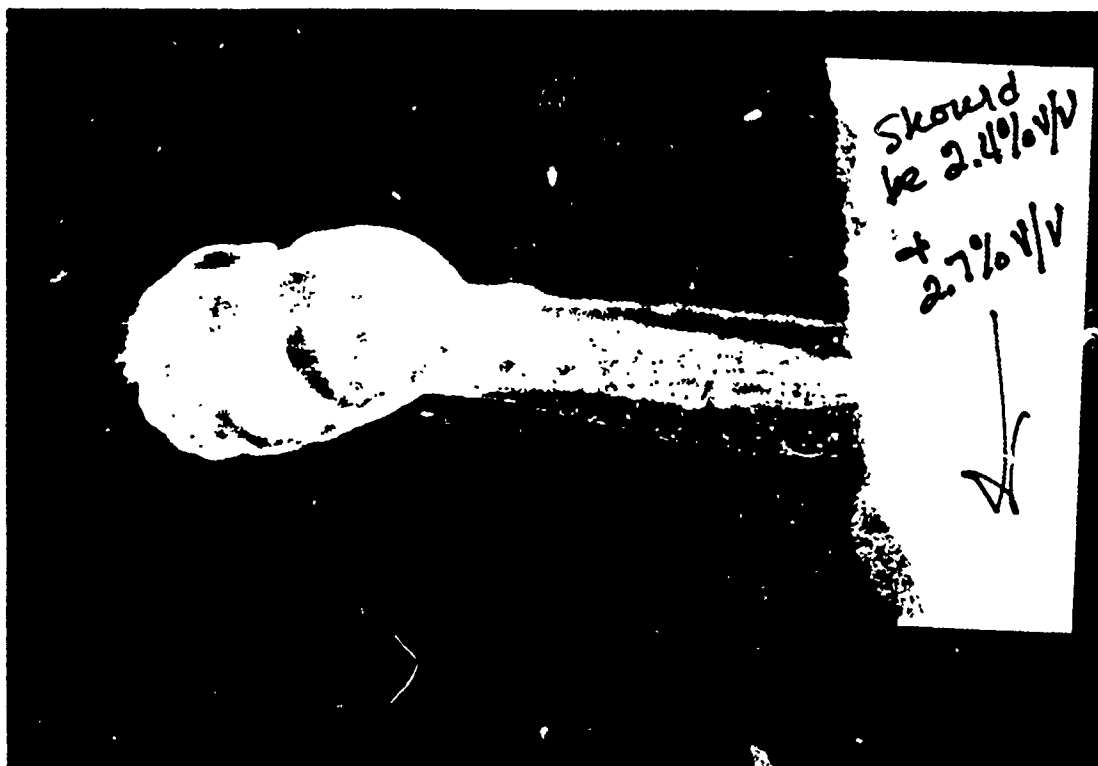


Plate 13a. Effects of a Medium Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 2.4 mg/ml Triethylene Glycol.



Plate 13b. Effects of a High Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2.7 mg/ml Triethylene Glycol.

Bantle, John A.

Acetone: Acetone can dissolve a large number of organic compounds but it is volatile. Acetone has been shown to potentiate hepatotoxicity induced by Trichloroethylene-carbon tetrachloride mixtures (41). While acetone may be the last solvent of choice, it may prove to be the only solvent that will carry some test compounds into solution. Shepard (34) does not discuss the developmental toxicity of Acetone.

Table 2 shows that the mean TI for Acetone is about 1.7. This is above the cutoff of 1.5 for a nonteratogen. The mean 96-hr LC50 and EC50 (malformation) is 2.19 and 1.29 v/v% respectively and the experiments replicated reasonably well considering the volatility of Acetone (Table 2). Figure 37 shows a representative mortality and malformation dose-response curve for Acetone. Note the obvious separation of the two curves which indicates that Acetone has weak teratogenic potential. The minimum concentration to inhibit growth was 1.25 v/v%. Figure 38 shows a representative growth inhibition curve for Acetone which is flat over a broad concentration range and then increasingly inhibits growth up to the 100% of the 96-hr LC50 level. Even at this concentration, the embryos are only inhibited about 20% of their growth. Plates 14 and 15 show the effect of Acetone on Xenopus development. Plate 14 is a concentration series seen from the side (14A) and ventral (14B) aspects. The embryo from the 0.9 v/v% (second from top) exposure concentration shows essentially no difference from the control (top). This would be near the final acetone solution concentration that would be used if acetone was employed as a carrier solvent. Plate 14 also shows that severe malformations occur only at about 2% concentration and that all organ systems are involved. Plate 15A shows a ventral view of an embryo exposed to 0.9% v/v Acetone. The only apparent change from controls is a slightly reduced gut coiling. This should be regarded not as a malformation but as a case of delayed development. Exposure to clean water and time would probably correct this situation. Plate 15B shows an embryo exposed to 1.5% v/v Acetone and there are indications of stress at this concentration. The choroid fissure of the eye has not yet completely fused at the ventral aspect and there is evidence of edema in the ocular and cardiac regions. These are modest defects, however, considering that 1.5% v/v Acetone is nearly 70% of the 96-hr LC50.

In summary, Acetone is not an ideal solvent because of its high teratogenic index. We have also observed that bacteria can utilize it as a carbon source. However, it may have to be employed for those test compounds that cannot be carried into solution any other way.

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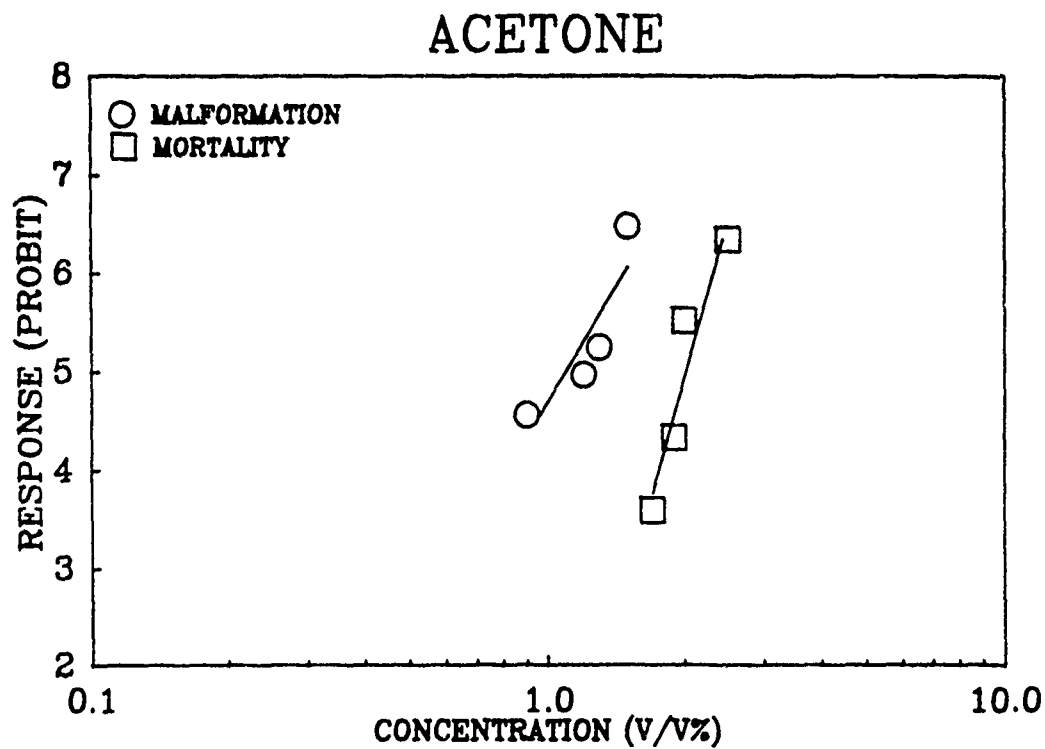


Figure 37. 96-h Mortality and Malformation Dose-Response Curves for Acetone Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

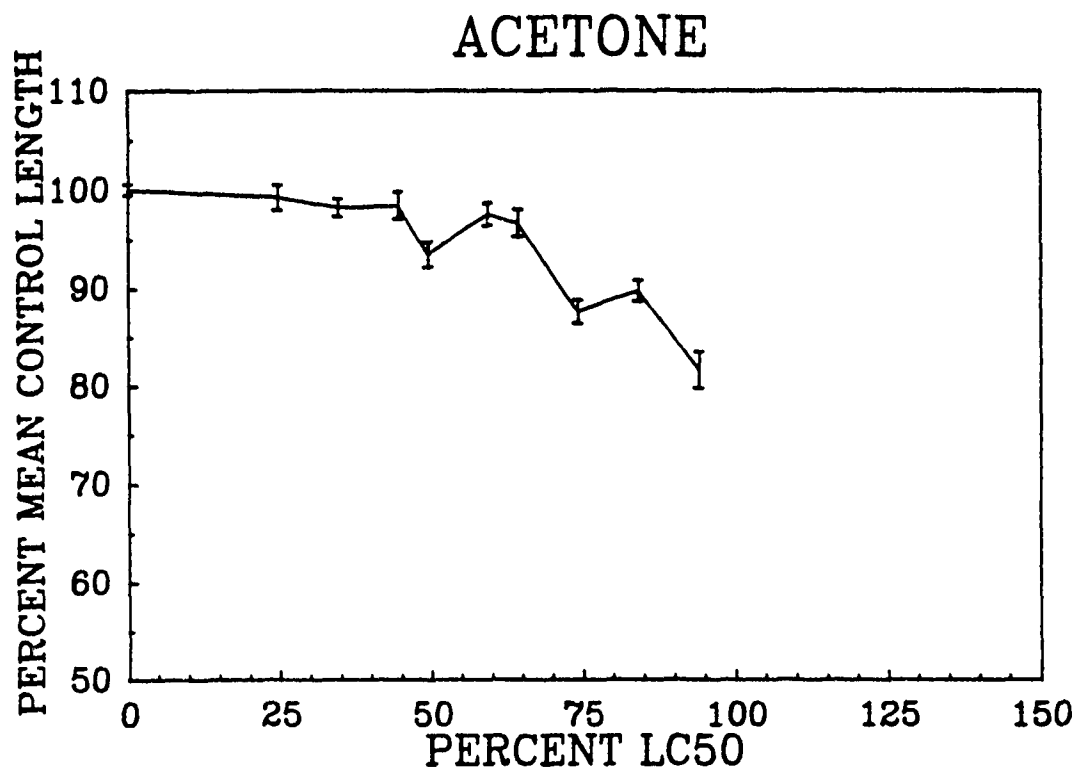


Figure 38. 96-h Growth Dose-Response Curve for Acetone Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 14A. Effects of Different Concentrations of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.9% v/v, 1.5% v/v, 2% v/v.



Plate 14B. Effects of Different Concentrations of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on cardiac and gut regions. From top to bottom: control, 0.9% v/v, 1.5% v/v, 2% v/v.



Plate 15A. Effects of a Low Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on cardiac and gut regions. Embryo exposed to 0.9% v/v Acetone.

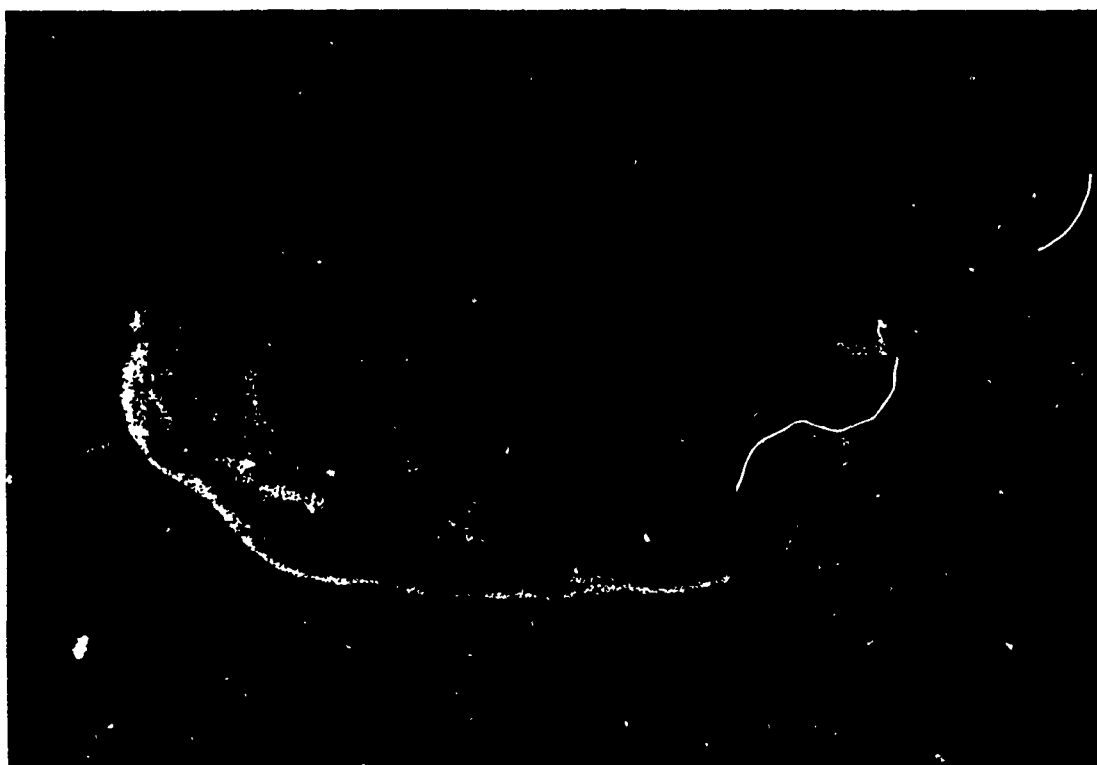


Plate 15B. Effects of a Medium Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 1.5% v/v Acetone.



Plate 16. Effects of a High Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2% v/v Acetone.

Bantle, John A.

Dimethyl Sulfoxide: Dimethyl Sulfoxide (DMSO) is a remarkably good solvent and has relatively low toxicity. DMSO should be listed as a variable negative in terms of its developmental toxicity (34). Caujolle et al. (42) reported teratogenic responses in rabbits, chickens, mice and rats when DMSO was administered at very high doses. However, at low doses few defects were seen. Juma and Staples (43) found increased resorptions in the rat embryo at high dose levels suggesting that DMSO is primarily embryotoxic. These variable results reported in the literature may be due to differences in dosage in the various studies. High doses of even nonteratogens can cause abnormal development in the few surviving offspring.

Table 2 shows the results of three definitive experiments on the effect of DMSO on Xenopus embryos. The mean TI was 1.4 while the mean 96-hr LC50 and EC50 (malformation) was 1.81 and 1.31% v/v respectively. Dumont reported a TI of 1.75 for DMSO so our values are not too far apart. The mean minimum concentration that inhibits growth was 1.38% v/v. There was excellent agreement among all three experiments. The results prove that a 1% v/v final concentration of DMSO could successfully be used as a carrier in FETAX (This concentration is safely below the MCIG). Figures 39 and 40 show the dose-response curves from the latter two definitive experiments listed in Table 2. The mortality and malformation curves are generally close together indicating low teratogenicity. There are a number of data points between the 0 and 100% effect levels for both endpoints and the data fit is close to the line. The NOEC is around 1.1 to 1.2% v/v for both endpoints. Figures 40 and 42 show the effects of DMSO on Xenopus growth. Figure 40 shows an indication of hormesis as embryos were longer after DMSO exposure than controls. This phenomenon was not borne out in Figure 42. Both Figures show that growth inhibition does not become a serious factor until well past 50% of the 96-hr LC50 concentration. After this point a severe reduction in growth is observed. Plates 17 and 18 show the typical effects of DMSO on Xenopus embryos after 96 hr of continuous exposure. Plate 17A shows a progression of increasing malformation and growth retardation in increasing concentrations of DMSO. There is little spinal kinking and edema. Facial, head and eye malformations occur at higher concentrations of DMSO. At 1.3% v/v DMSO, the effects are slight (Plates 17A&B). A reduction in embryo size and gut coiling are seen. At 1.7% v/v DMSO, there is evidence of some edema as well as problems in gut coiling and development of the facial region (Plate 18A). At 2% v/v DMSO (Plate 18B), the embryo is severely malformed with all major organ systems being involved.

In summary, DMSO is an excellent solvent and exhibits low teratogenicity in FETAX. There should be no problem in using 1% v/v final concentrations of DMSO as a carrier unless further studies show real interaction problems.

Bantle, John A.

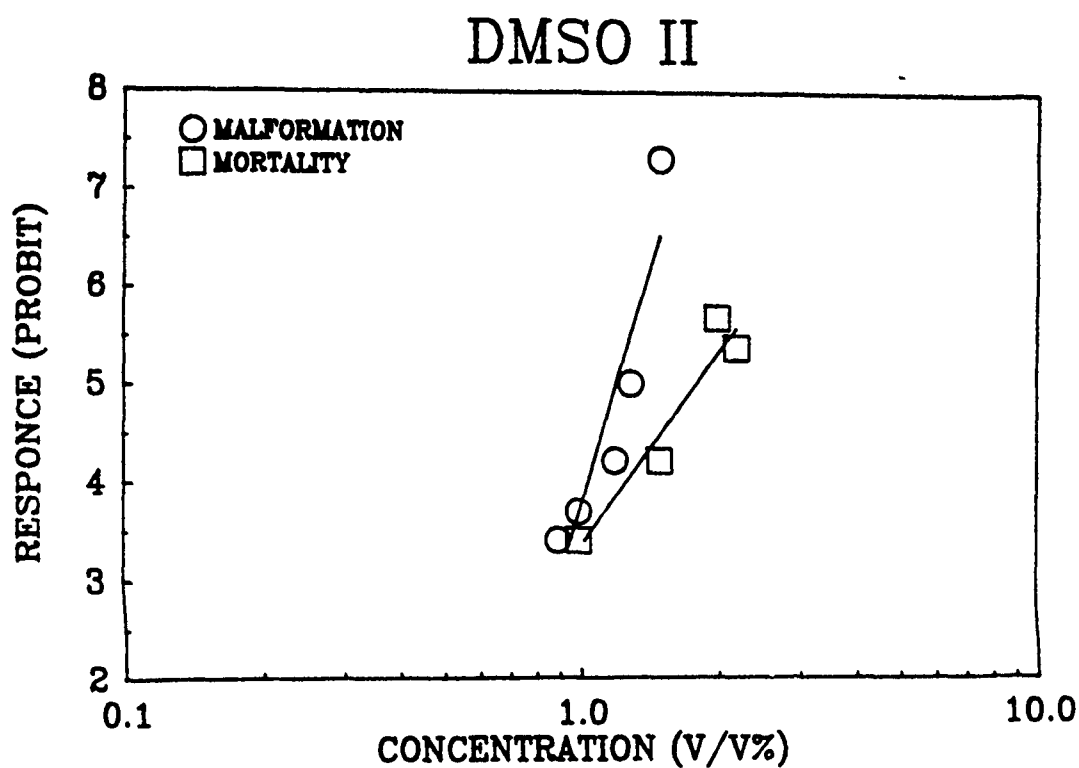


Figure 39 96-h Mortality and Malformation Dose-Response Curves for Dimethyl Sulfoxide Definitive Test #2. The curve includes only those points used in producing the dose-response curves although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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DMSO II

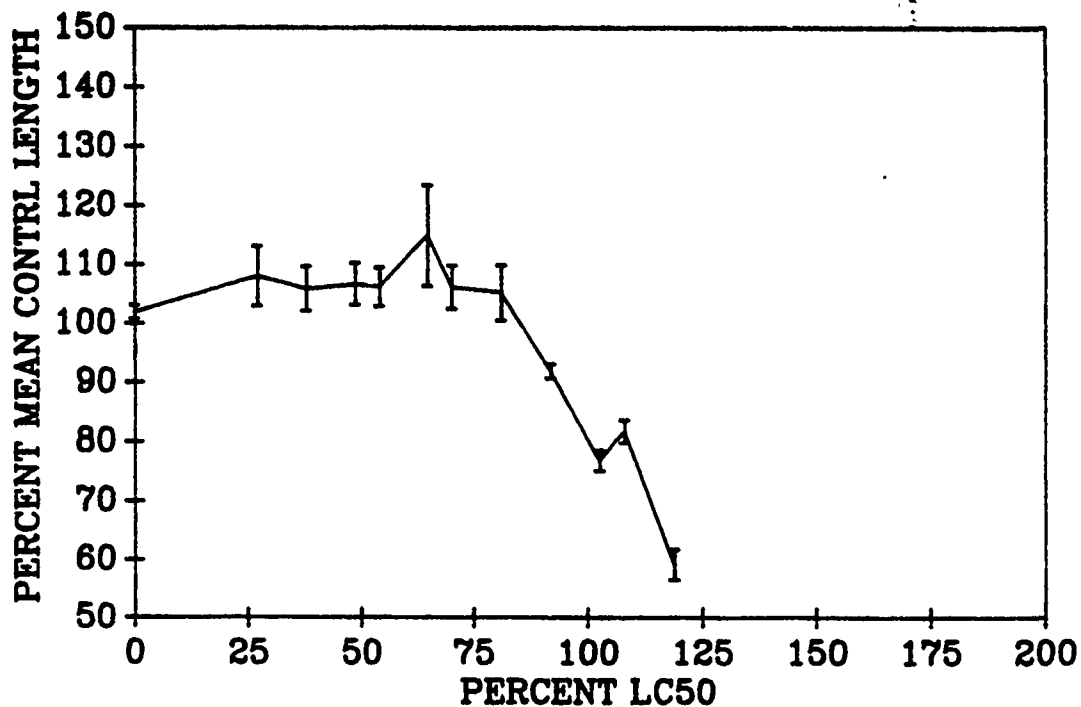


Figure 40. 96-h Growth Dose-Response Curve for Dimethyl Sulfoxide Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Bantle, John A.

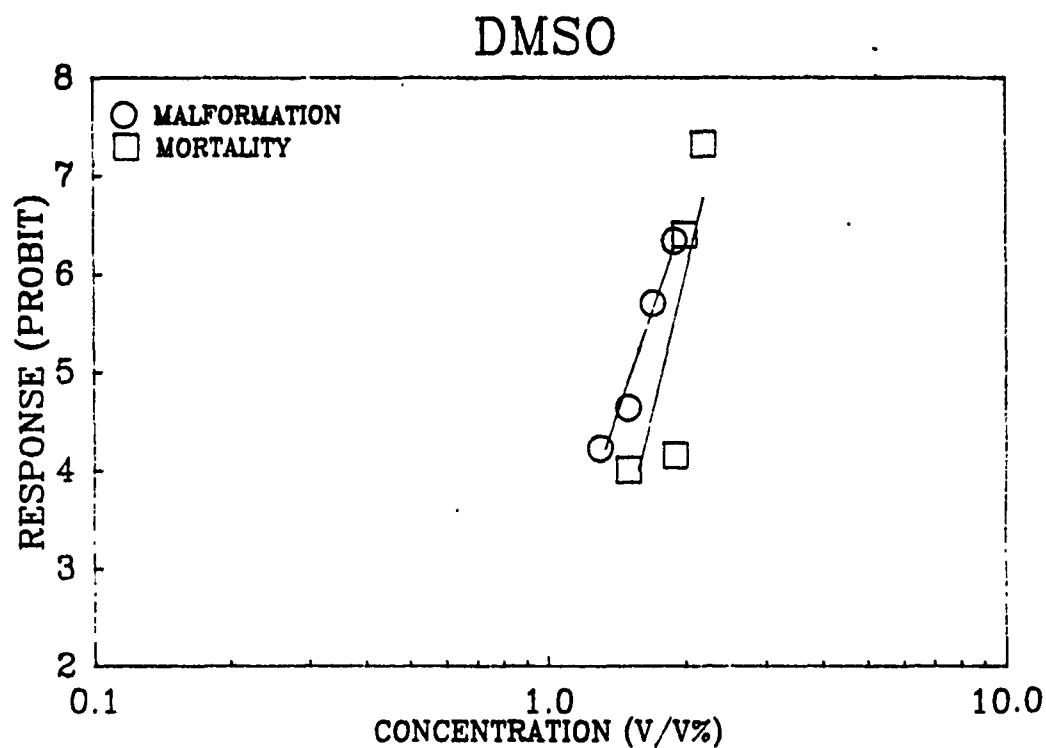


Figure 41 . 96-h Mortality and Malformation Dose-Response Curves for Dimethyl Sulfoxide Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

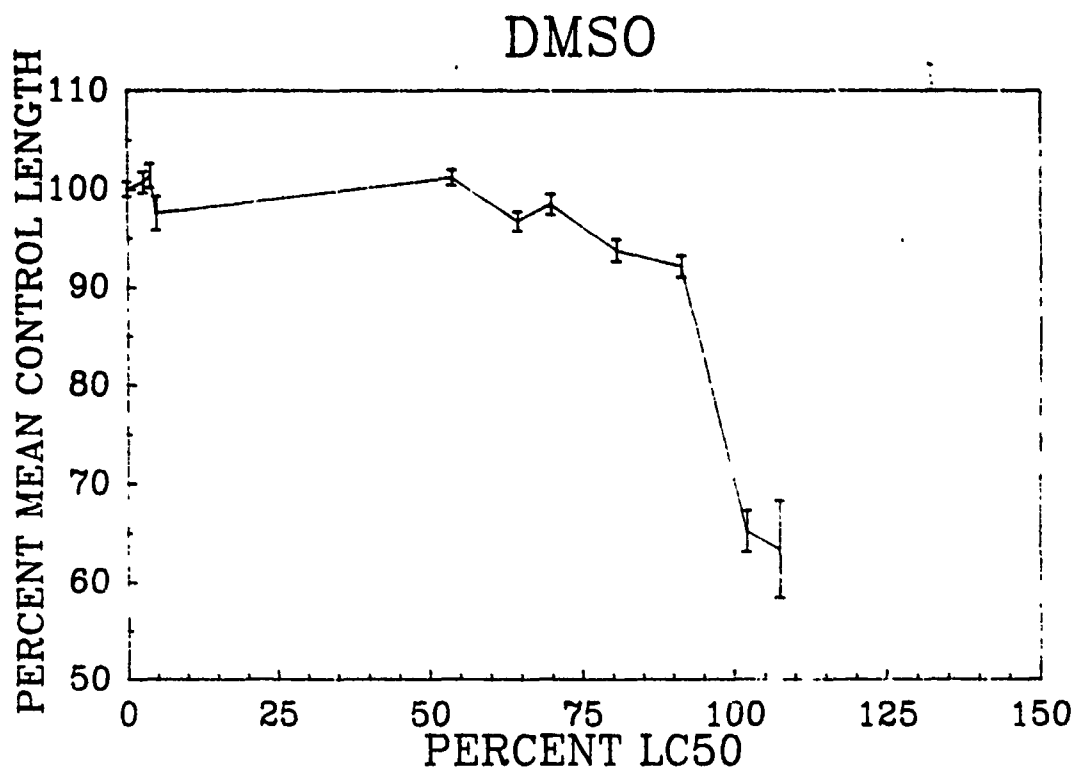


Figure 42. 96-h Growth Dose-Response Curve for Dimethyl Sulfoxide Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 17A. Effects of Different Concentrations of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 1.3% v/v, 1.7% v/v, 2% v/v.

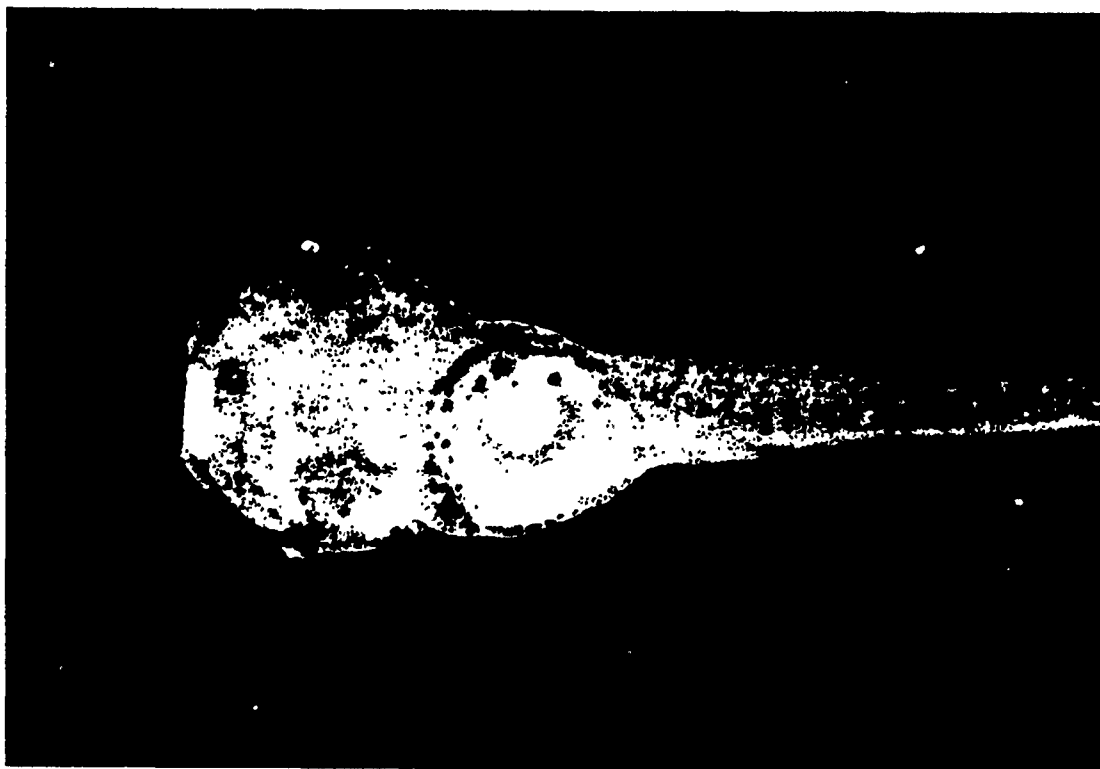


Plate 17B . Effects of a Low Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 1.3% v/v Dimethyl Sulfoxide .

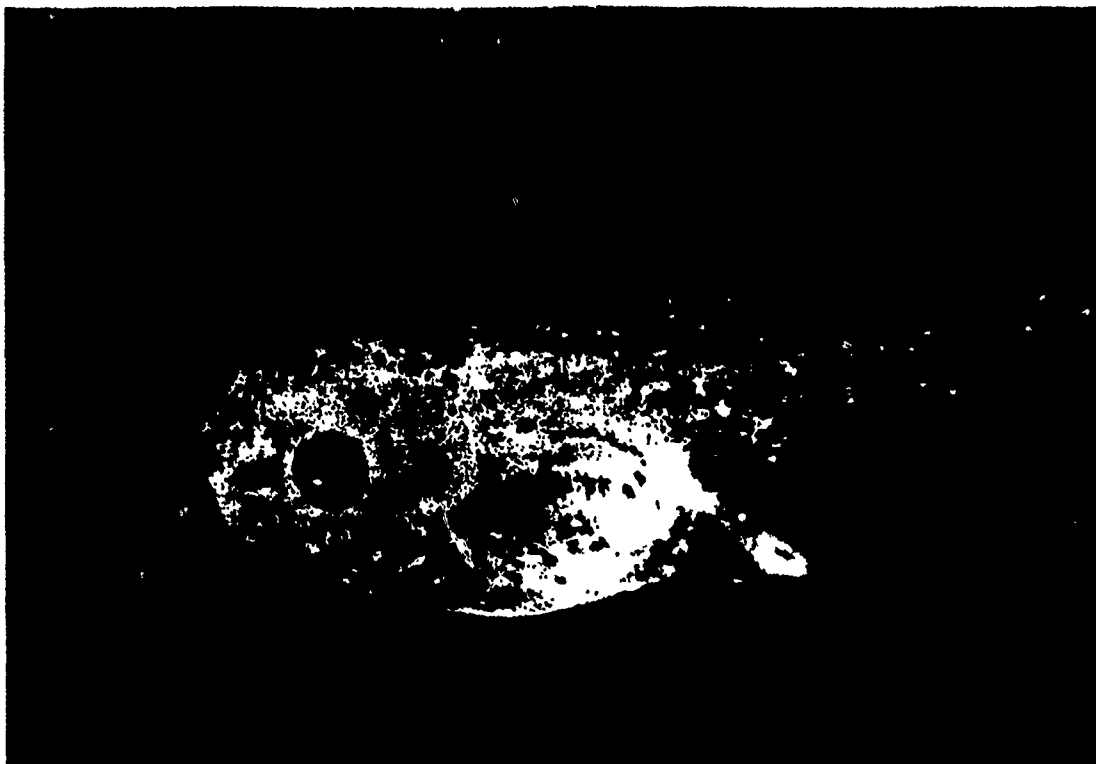


Plate 18A. Effects of a Medium Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 1.7% v/v Dimethyl Sulfoxide.

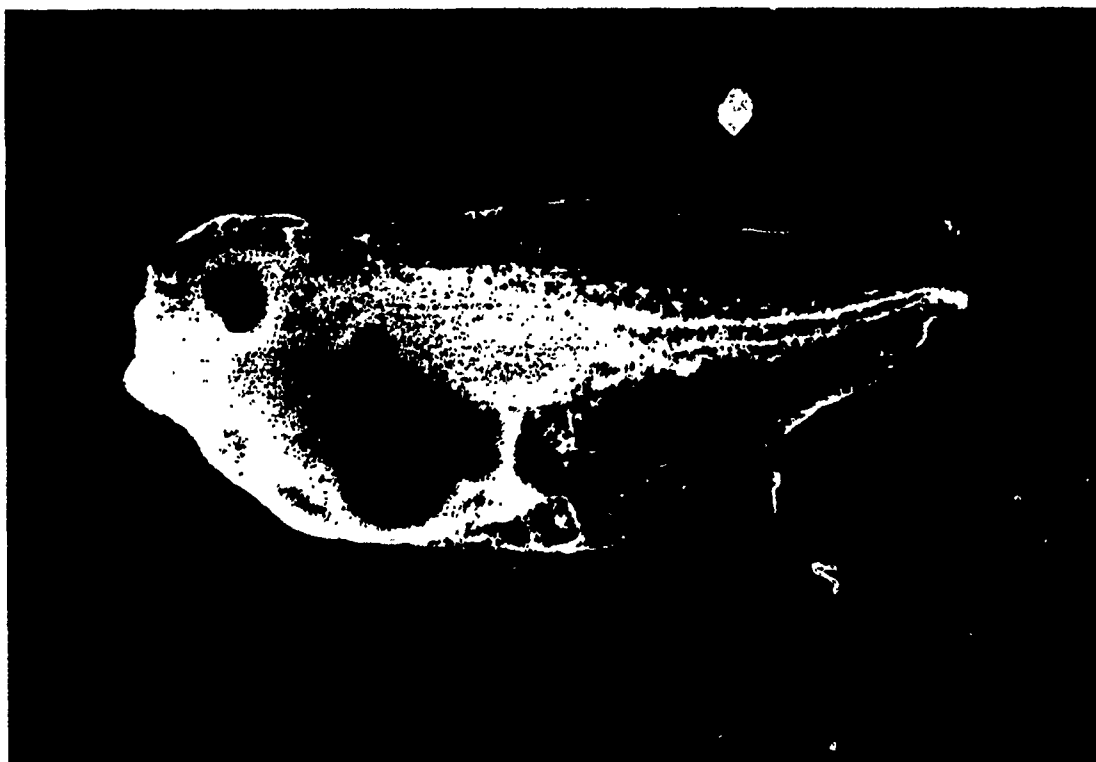


Plate 18B. Effects of a High Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2% v/v Dimethyl Sulfoxide.

Bantle, John A.

Trans-retinoic acid: Work on this compound is being supported by the Oklahoma Center for the Advancement of Science and Technology (OCAST). The data is presented here in order to present a clear idea as to the nature of the upcoming solvent interaction study. trans-retinoic acid is a classic teratogen with numerous listings in Shepard (34). It is listed by Smith et al. (22) as a strong teratogen that is quite insoluble in water. Despite its insolubility in water, enough of the compound gets into solution to have an effect in FETAX without the use of a carrier. This made it an ideal compound to use in the carrier interaction study because results of retinoic acid alone vs retinoic acid plus carrier could be easily compared. Table 1&2 shows that three different teratogens (Trans-retinoic acid, Me-mercury chloride and 6-Aminonicotinamide) of differing solubilities and teratogenic actions were selected for this study as well as Trichloroethylene, a nonteratogen, of low water solubility.

Table 1 shows that there was difficulty in repeating the dose-response tests for Trans-retinoic acid. The mean TI was close at 10.5 but that in the first definitive experiment the 96-hr LC50 was only 0.246 ug/ml versus 0.5 for the second definitive. Upon inspection of the data in Table 1 both the 96-hr LC50 and EC50(malformation) of the first test were approximately 2 fold lower than the second test. Although we checked the concentrations of each stock solution spectrophotometrically, it is possible an error was made in weighing or dissolving the insoluble Trans-retinoic acid. Previous range tests also showed this type of variability for this compound. The high TI of 10.5 proved that Trans-retinoic acid was a strong teratogen in FETAX and this result agrees well with the mammalian literature. Sabourin and Faulk (14) obtained a TI >2.6 for Trans-retinoic acid while Dumont obtained a TI of 6.6. It should be remembered that Sabourin and Faulk only reported ranges of TI and not actual values. Figures 43 and 45 show the mortality and malformation dose-response curves. They are widely separated, have essentially the same slope and there are a good number of data points that make up each curve. The mean MCIG is 0.07 which is 28% of the 96-hr LC50. From Figure 44 and 46 it can be seen that growth inhibition increases rapidly faster about 25% of the 96-hr LC50. The maximum amount of growth inhibition is about 35% (65% of control length). Thus, the growth inhibition data is supportive of the hypothesis that Trans-retinoic acid is a teratogen. Plates 19 and 20 show the effects of increasing Trans-retinoic acid concentrations on Xenopus development (See 19A for overview). Plate 19B shows the effect of dose near the EC50(malformation) had on development. Minor malformations were seen in the face, eye and gut region. At 0.2 ug/ml severe malformations were seen that involve all major organ systems (Plate 20A). Curiously, The tail was not kinked. At very high concentrations (0.5 ug/ml), very severe malformations were observed. This embryo was alive at 96 hr but severely malformed. The eye is cyclopic and the gut is nearly straight. At this concentration, nearly half of the embryos were still alive.

Despite the variability in results, Trans-retinoic acid is an ideal compound with which to test the possibility of solvent interactions on Xenopus development. The question arises as to whether the same defects will be seen if there is interaction or whether new malformations will be caused.

Bantle, John A.

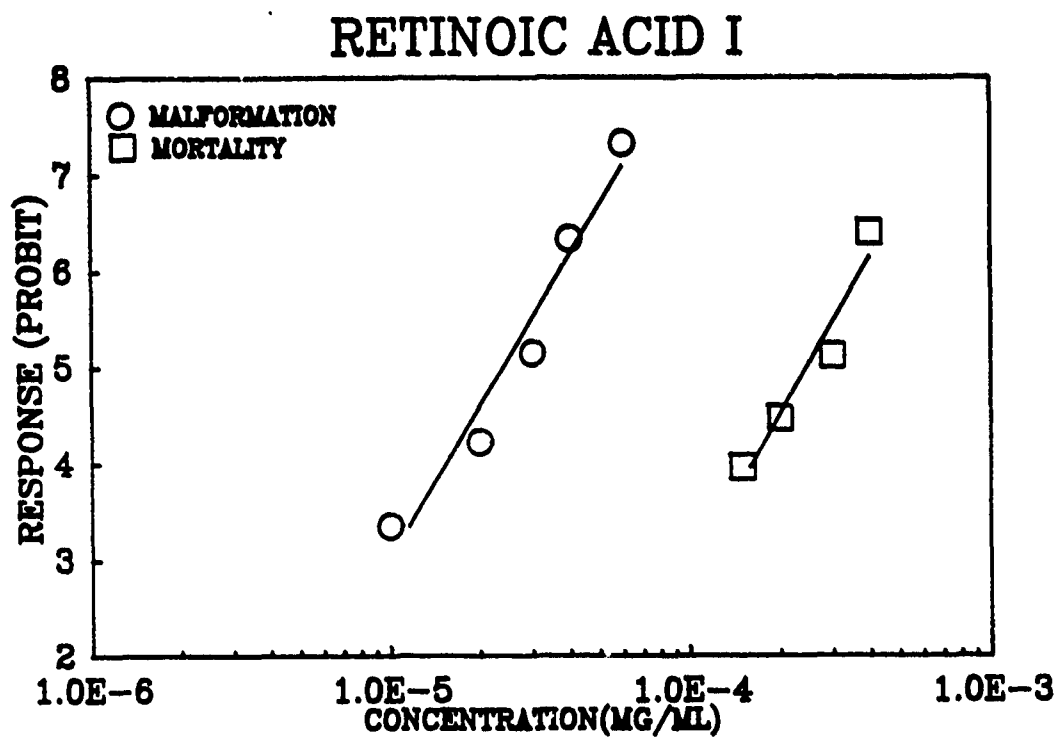


Figure 43. 96-h Mortality and Malformation Dose-Response Curves for Retinoic Acid, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

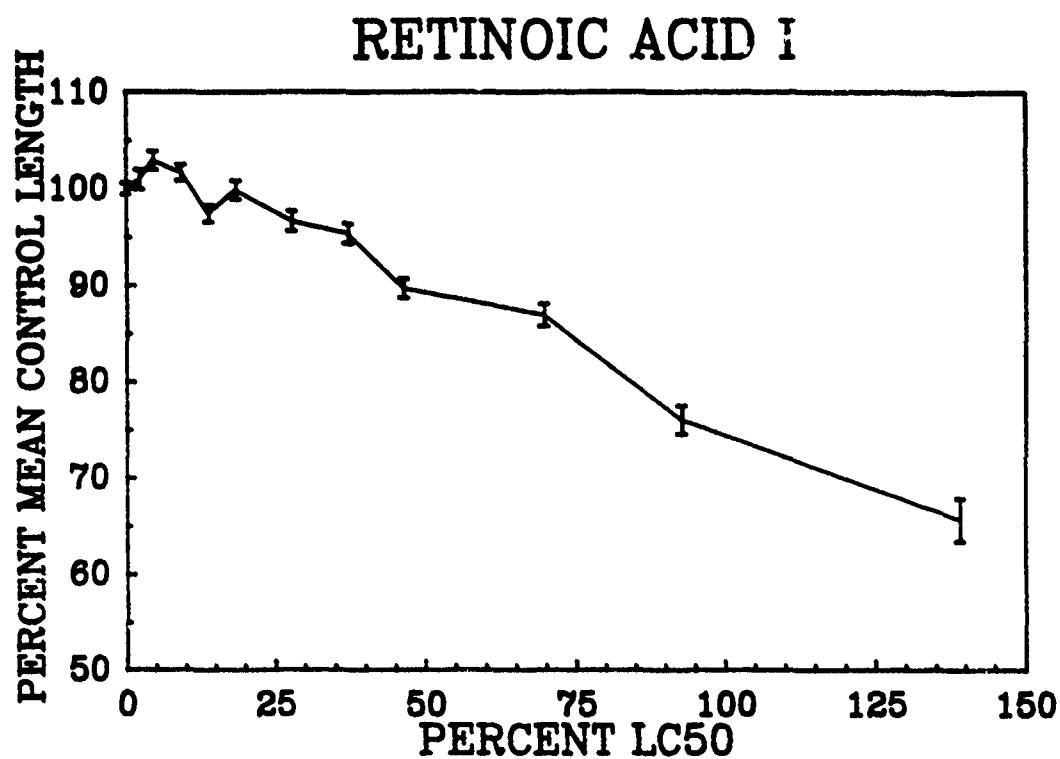


Figure 44. 96-h Growth Dose-Response Curve for Retinoic Acid, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Bantle, John A.

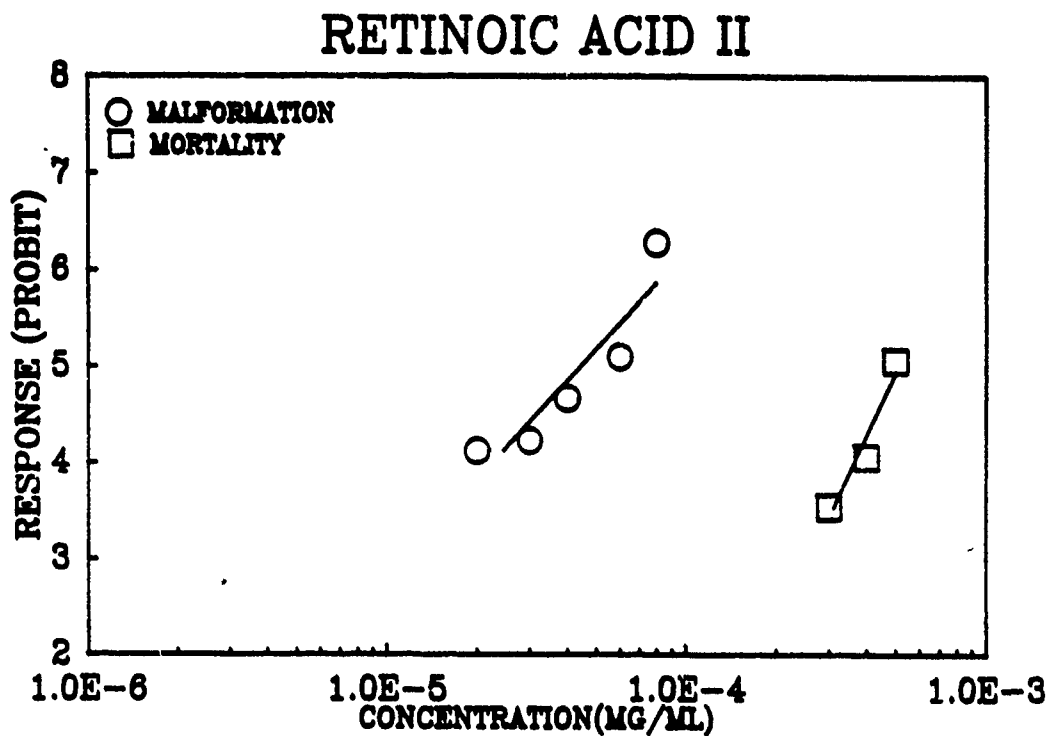


Figure 45. 96-h Mortality and Malformation Dose-Response Curves for Retinoic Acid, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

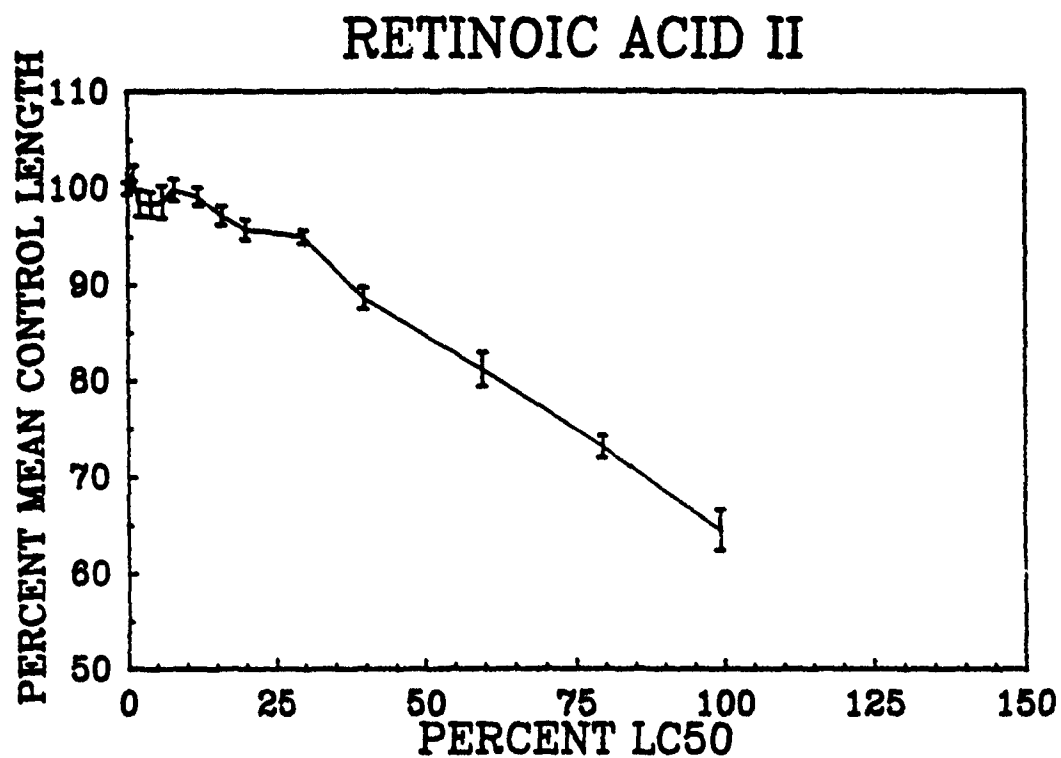


Figure 46. 96-h Growth Dose-Response Curve for Retinoic Acid, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 19A. Effects of Different Concentrations of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.02 ug/ml, 0.2 ug/ml, 0.5 ug/ml.

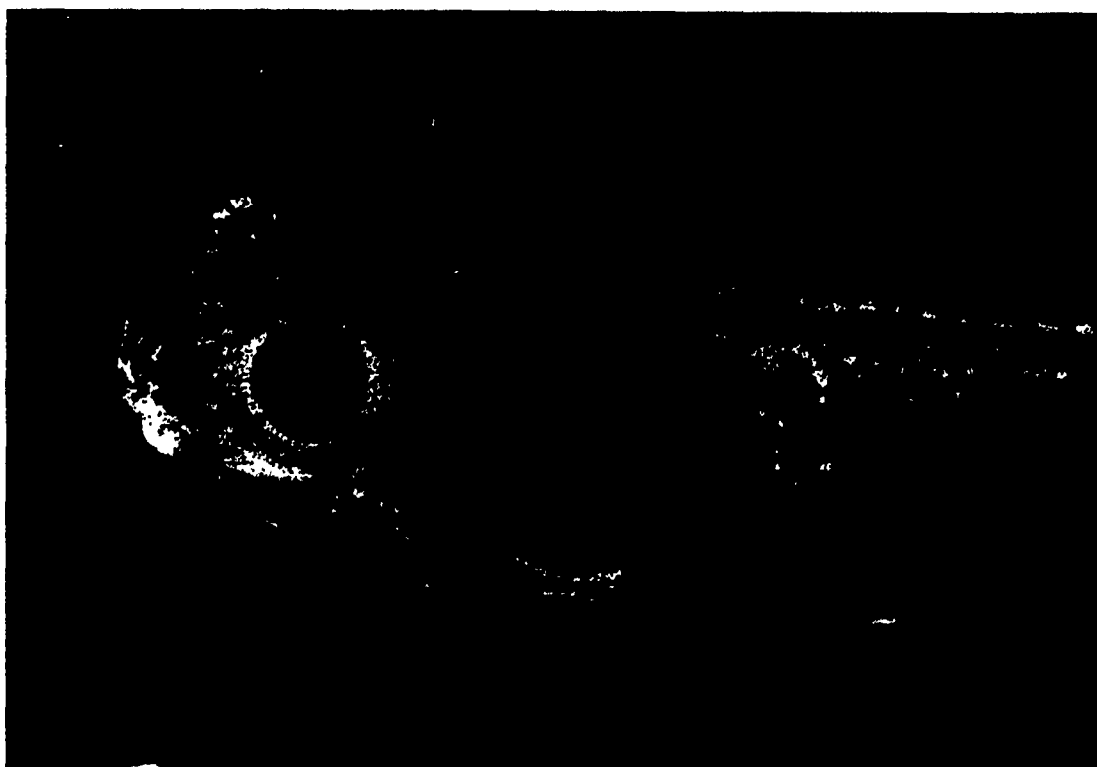


Plate 19B. Effects of a Low Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.02 ug/ml Trans-retinoic acid.



Plate 20A. Effects of a Medium Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.2 ug/ml Trans-retinoic acid.

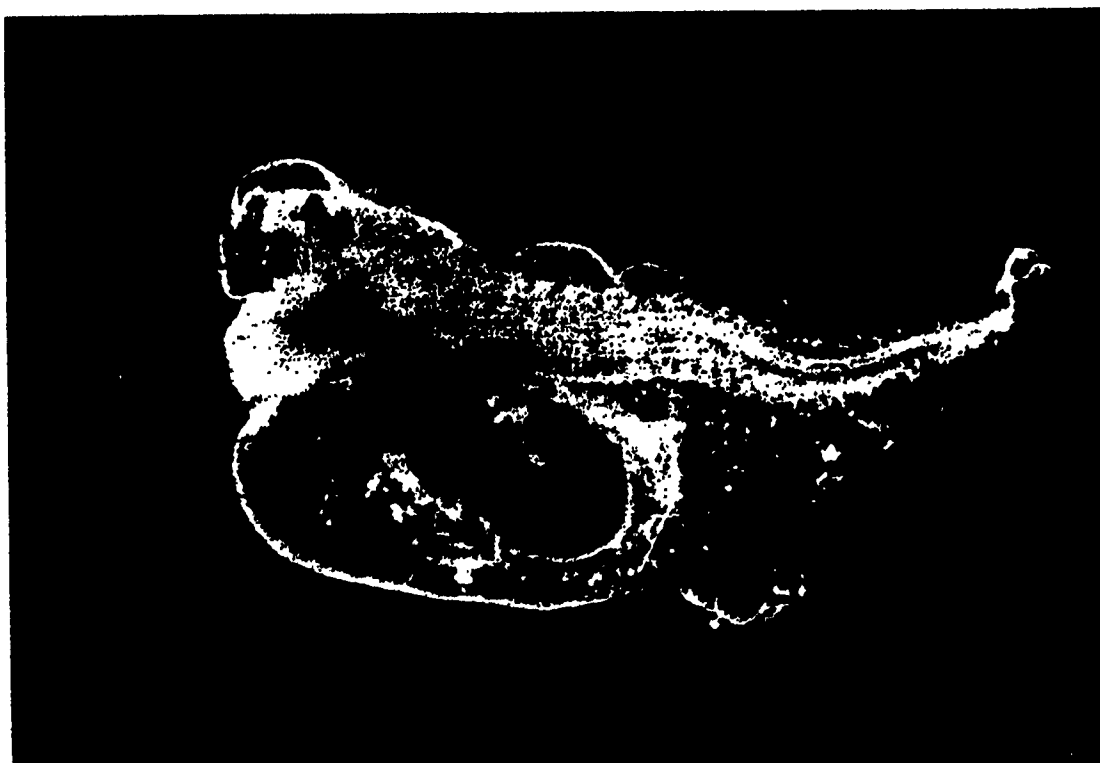


Plate 20B. Effects of a High Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.5 ug/ml Trans-retinoic acid.

Methylmercury Chloride: is a known mammalian teratogen and is similar in effect to mercury but passes through membranes much more easily. Methylmercury Chloride (MMC) is known to effect the central nervous system of animals. MMC was chosen to test solvent interactions because of its relatively insolubility in water.

Table 1 shows that MMC has a TI of approximately 3 which is teratogenic. However this TI indicates a moderate teratogen, and MMC is considered highly teratogenic. MMC is highly embryo toxic in FETAX. Figures 47,49,51 show the dose-response curves performed for MMC. There are a number of points between the 0 and 100% effect levels for both endpoints and the data fit is close to the regression line. There is a clear separation between the two curves. The NOEC is around 0.008 averaged over the three experiments for Malformation. The NOEC for mortality is approximately 0.08. Figures 48,49,52 show effects of MMC on Xenopus growth. Figures 48 and 50 indicate hormesis but only a slight increase in growth is seen. Figure 52 does not show any hormesis. All figures show no inhibition of growth until or after 50% of the LC50 value. Plate 21 shows the typical malformations seen with MMC. Plate 21 also shows a progression of increasing malformations and growth retardations in increasing concentrations of MMC. Facial malformations and edema occur at low concentrations of MMC. As concentration increases effects on brain and spinal column can also be seen. At the highest concentrations most organ systems are effected.

In summary, MMC is extremely toxic to embryos but has a surprisingly low TI. Although severe malformations are seen at high concentrations usually the embryos die before 96 hours.

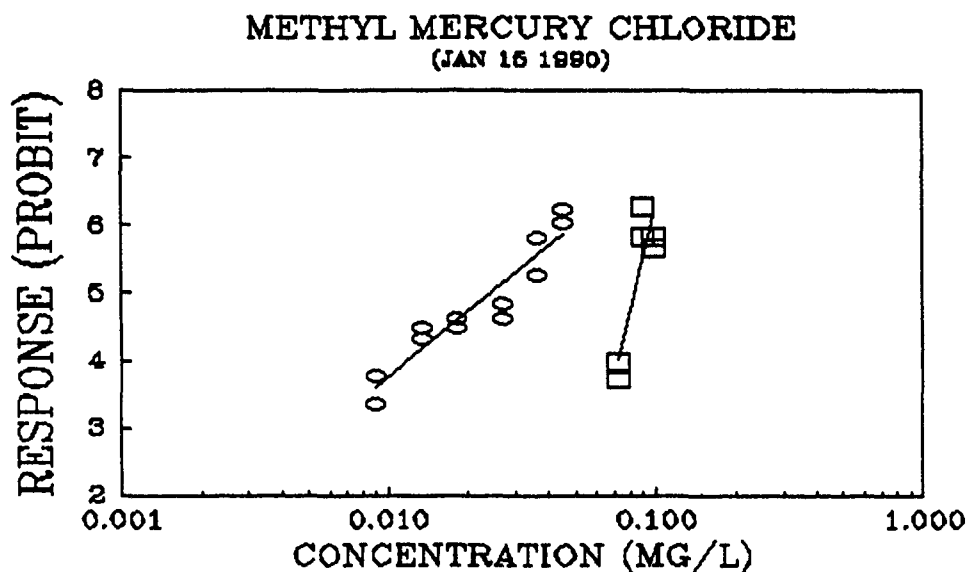


Figure 47 The 96-h Mortality and Malformation Dose-Response Curves for Methylmercury Chloride Definitive Test #1. The curve only includes those points used in producing the dose-response curves although other concentrations were tested as well (See: Appendix). Each point represent an experimental unit (one dish of 20 embryos).

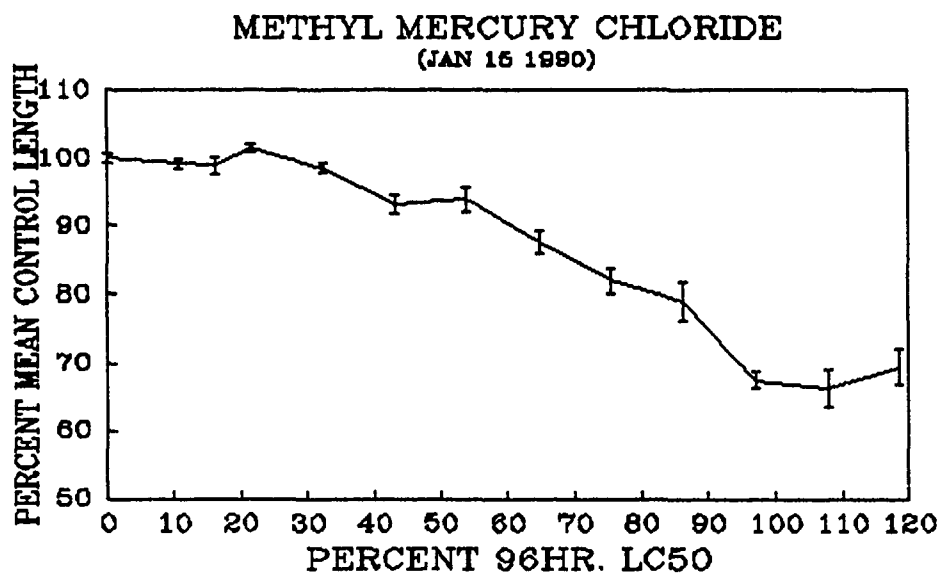


Figure 48. The 96-h Growth Dose-Response Curve for Methylmercury Chloride Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

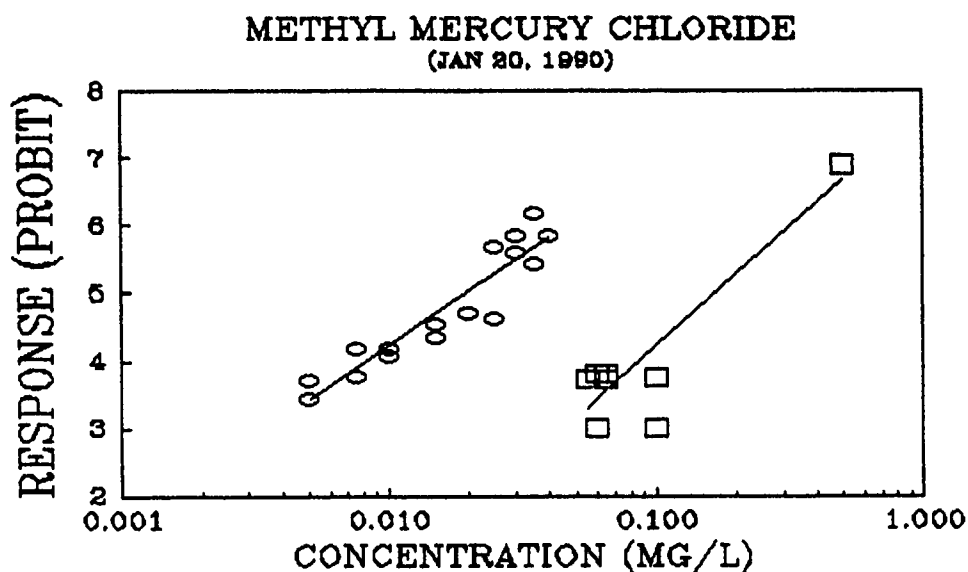


Figure 49. The 96-h Mortality and Malformation Dose-Response Curves for Methylmercury Chloride Definitive Test #2. The curve only includes those points used in producing the dose-response curves although other concentrations were tested as well (See: Appendix). Each point represent an experimental unit (one dish of 20 embryos).

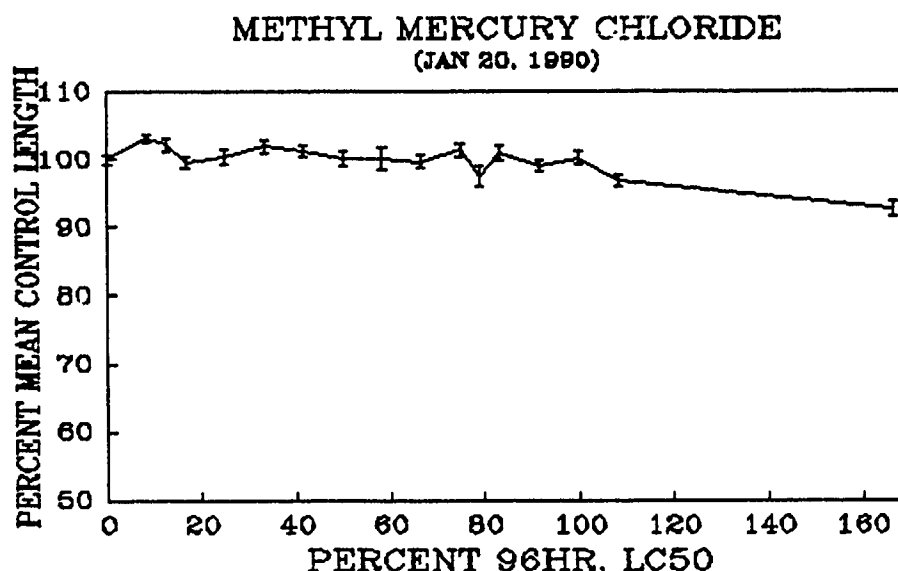


Figure 50. The 96-h Growth Dose-Response Curve for Methylmercury Chloride Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

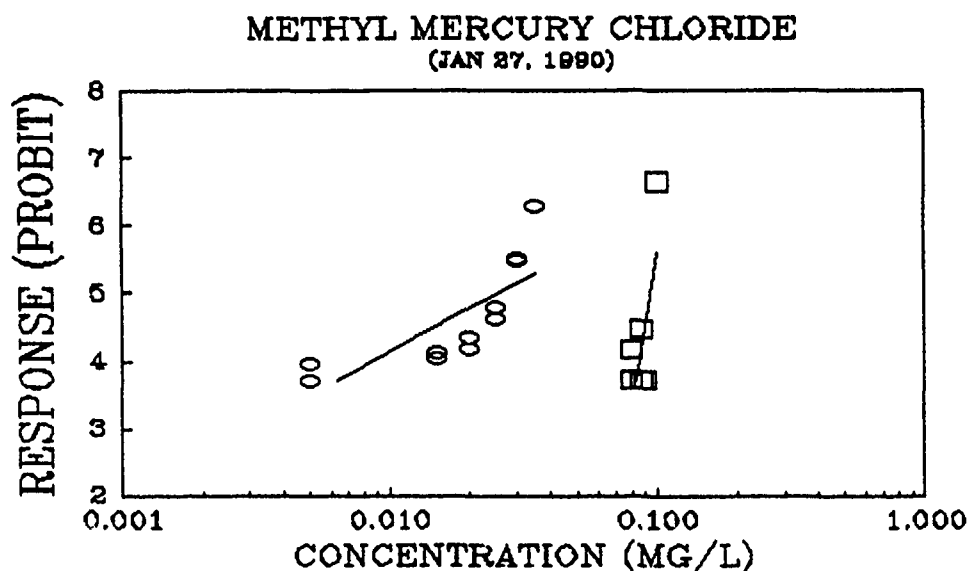


Figure 51. The 96-h Mortality and Malformation Dose-Response Curves for Methylmercury Chloride Definitive Test #3. The curve only includes those points used in producing the dose-response curves although other concentrations were tested as well (See: Appendix). Each point represent an experimental unit (one dish of 20 embryos).

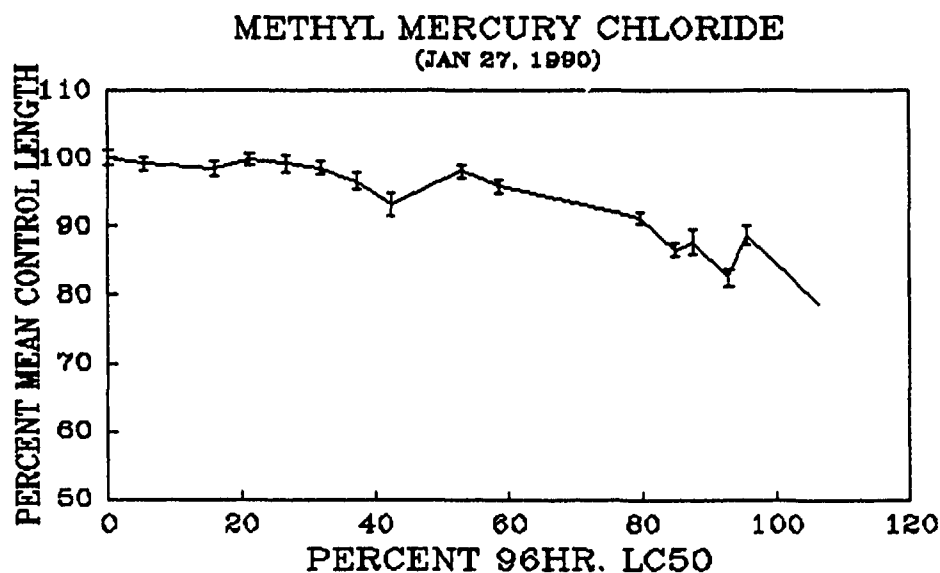


Figure 52. The 96-h Growth Dose-Response Curve for Methylmercury Chloride Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

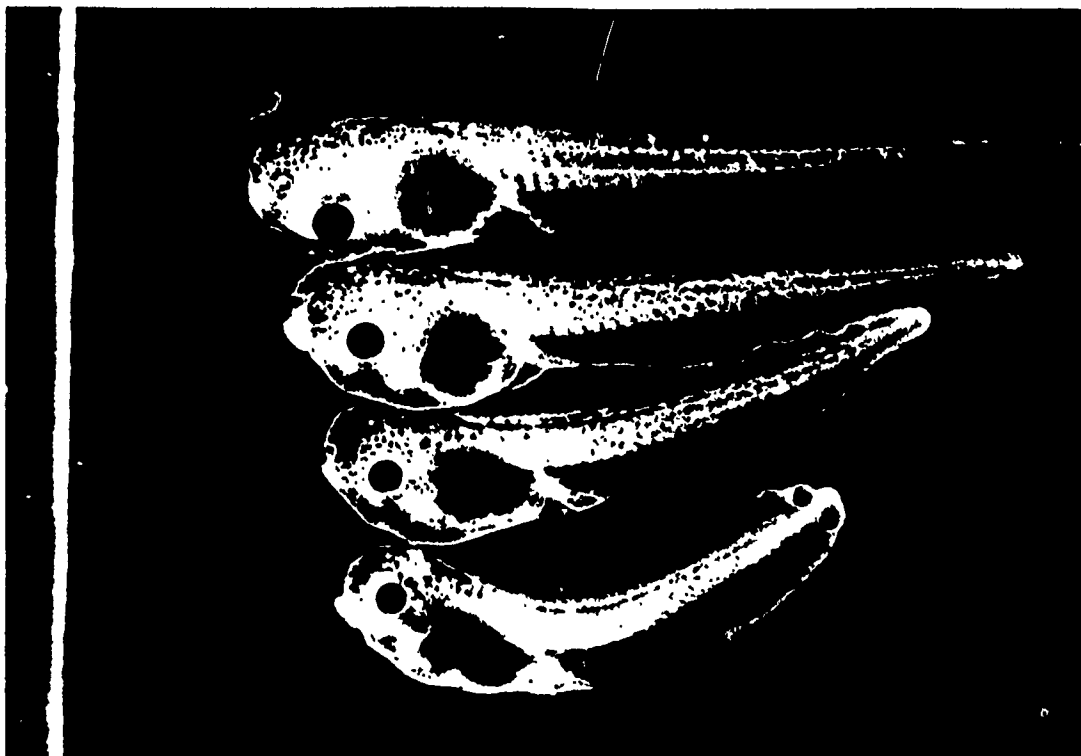


Plate 21 Effects of Different Concentrations of Methylmercury Chloride on Xenopus Development. Control embryos achieved stage 46 at the end of the 96-h exposure period. Side view presented to show effect on brain eye and spinal cord. From top to bottom: Control, 0.012 mg/L, 0.025 mg/L, and 0.04 mg/L.

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D. Phase IV

BUSULFAN

Busulfan is used in treatment of myelogenous leukemia in humans. It can also induce severe bone marrow hypoplasia. Teratogenicity studies have been done in the rat and mouse. At low doses, it produces a wide range of teratogenetic abnormalities in the rat.

Busulfan does not readily dissolve in FETAX. It was dissolved in acetone and then added to FETAX to make a final concentration of acetone of 1% (v/v). One range test was completed. Mortality rates were low in all concentrations. We can only estimate the LC50 as being >0.20 mg/ml. The malformation rates increased with increasing concentration. The EC50 was found to be 0.16 mg/ml using the Litchfield-Wilcoxon test. Only the Malformation Dose-Response curve could be plotted as shown in Figure 53. Common malformations were seen in the gut and face regions. Edema was observed in the cardiac, abdominal and facial regions. Also, severe malformations were recorded in the higher concentrations.

Solvent studies were conducted on Busulfan. Since the highest concentration that could be dissolved in a 1% acetone solution in FETAX without precipitating out was 0.2 mg/ml, we tried other solvents. We dissolved 50 mg of Busulfan in 2 mls of triethylene glycol. This was added to 98 mls of FETAX. The compound came out of solution. Using dimethyl sulfoxide (DMSO), we dissolved 500 mg in 1.2 mls of DMSO and brought it up to 100 mls with FETAX. The compound precipitated out. With 100 mg of Busulfan added to 1.2 mls of DMSO and brought up to 100 mls with FETAX, the compound precipitated out. Lastly, 50 mg of Busulfan was added to 1 ml of DMSO and brought up to 100 mls with FETAX. This was not successful either. Since a higher concentration of solvent is needed to increase the concentration of the compound in FETAX and these solvent concentrations are greater than what the ASTM Guide dictates, further testing was stopped.

The results gathered indicate a TI >1.25 . The severity and type of malformations indicate Busulfan to be a teratogen.

BUSULFAN

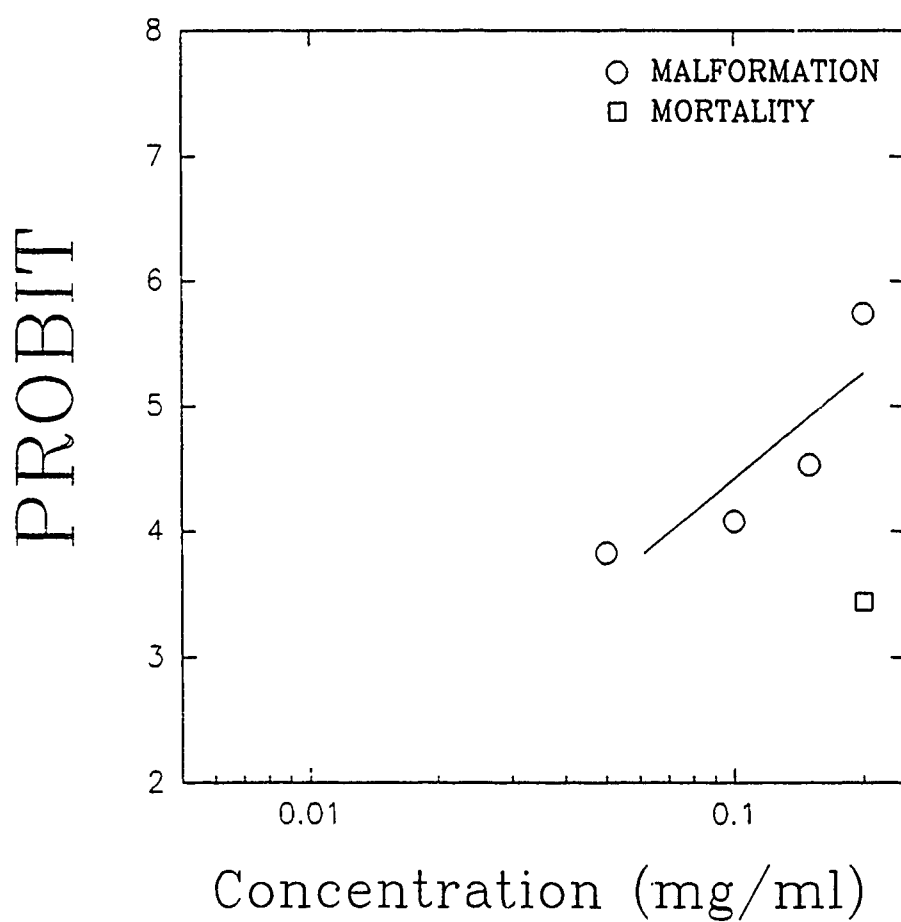


Figure 53. 96 h Mortality and Malformation Dose-response curves for Busulfan Range Test. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.



Plate 22. Effects of different concentrations of Busulfan on Xenopus development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on face, gut and notochord. From top to bottom: control, 0.05 mg/ml, 0.10 mg/ml, 0.15 mg/ml, 0.20 mg/ml.

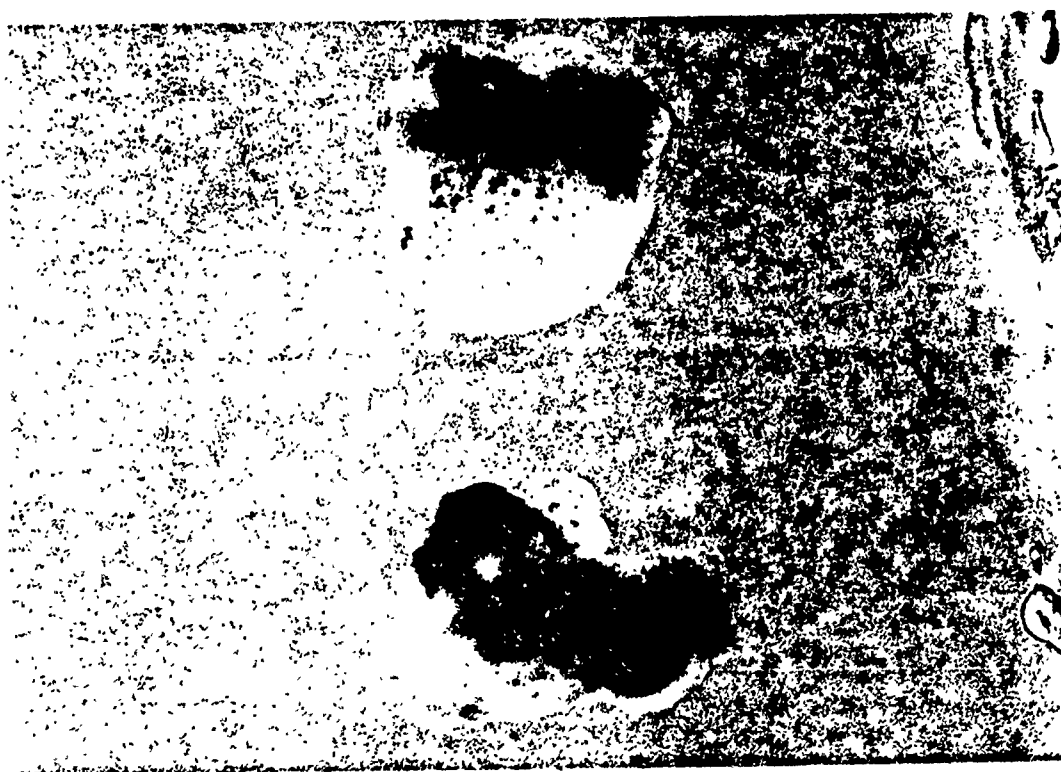


Plate 23. Severe abnormalities observed at medium concentrations. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view shows effect on abdominal region, face and notochord. From top to bottom: 0.10 mg/ml, 0.15 mg/ml.

Furazolidone

Furazolidone is on the Flint List. It is used as an antibiotic agent.

A range test gave a EC50 of 0.012 mg/ml. A LC50 was estimated to be approximately >0.018 mg/ml. The T.I. was estimated to be >1.5. The mortality curve in Figure 54 was figured from a transformation table to find the corresponding probit values. The most common malformations were seen in the gut, notochord and face.

The first definitive test gave a EC50 of 0.0109 mg/ml. The LC50 was estimated to be 0.015 mg/ml. This gives an approximate T.I. of 1.38. The most common malformations were in the gut, notochord and face. The mortality curve in Figure 56 was figured from a transformation table to find the corresponding probit values.

A second definitive test gave a EC50 of 0.00711 mg/ml and a LC50 of 0.014 mg/ml. This gave a T.I. of 1.97. The malformations most observed were gut, edema and face.

From this data, we conclude that Furazolidone is a teratogen.

FURAZOLIDONE

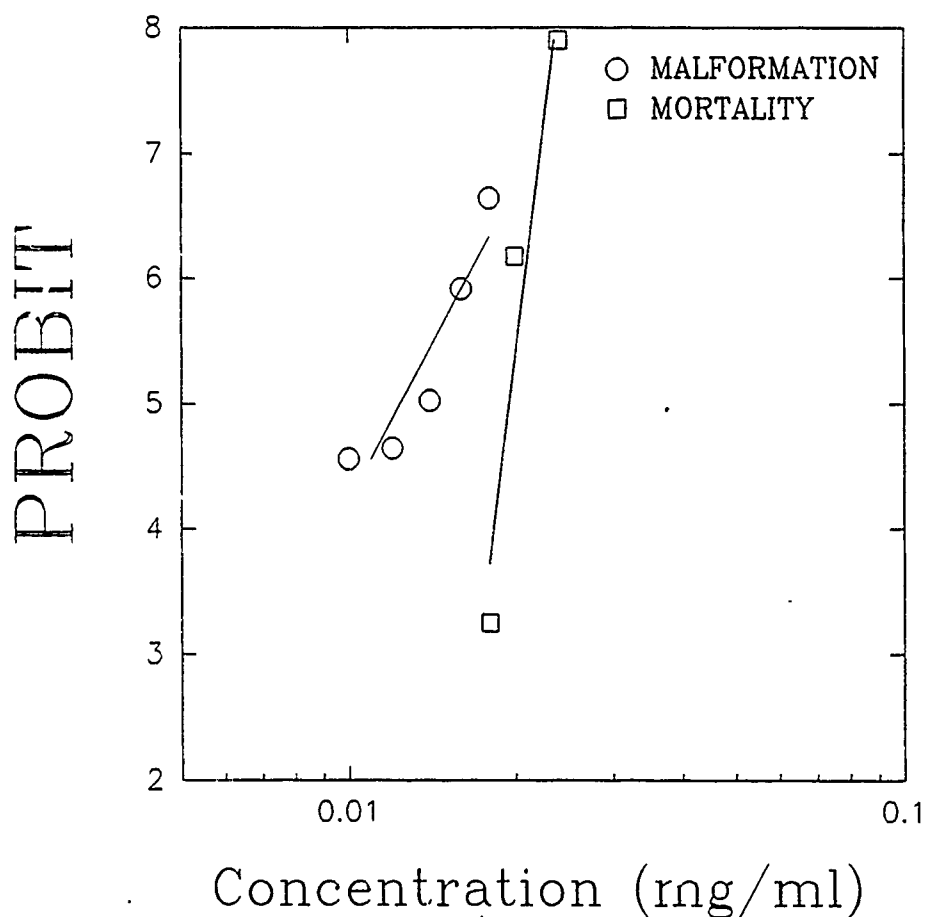


Figure 54 96 h Mortality and Malformation Dose-Response Curves for Furazolidone Range Test. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

FURAZOLIDONE

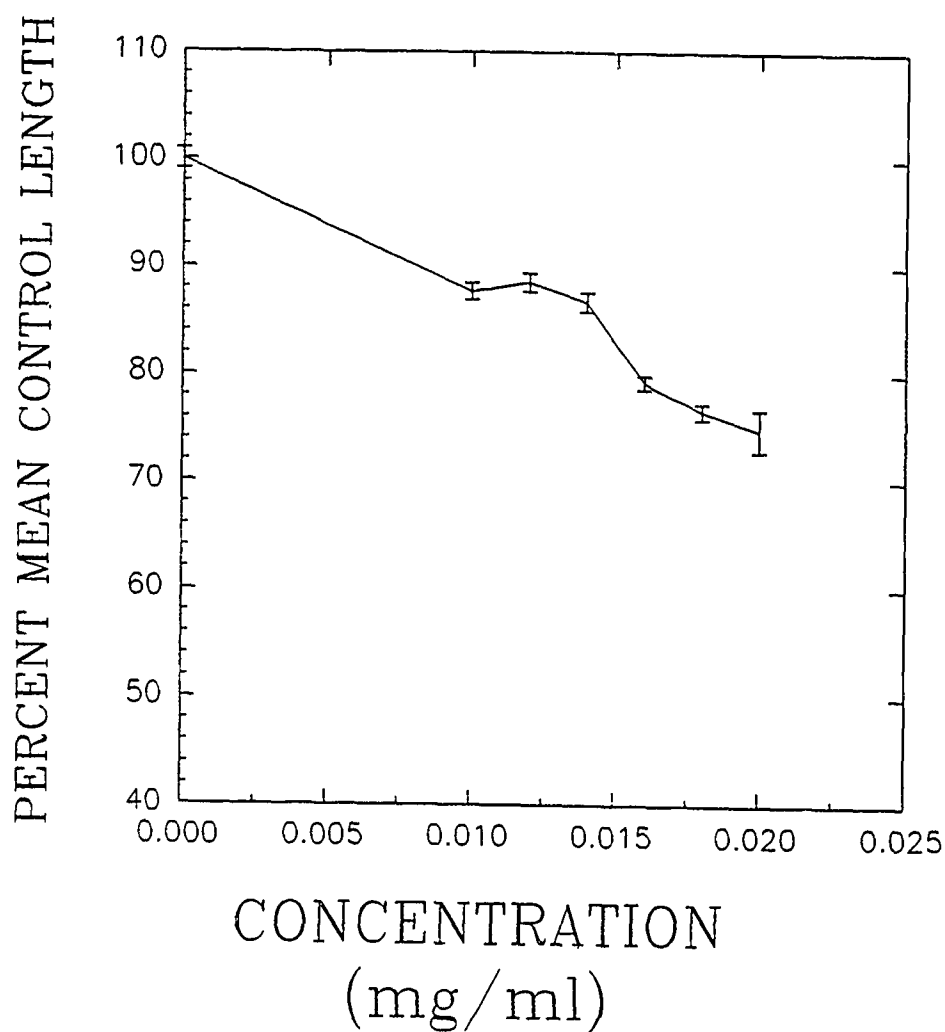


Figure 55 96 h Growth Dose-Response Curve for Furazolidone Range Test.

FURAZOLIDONE

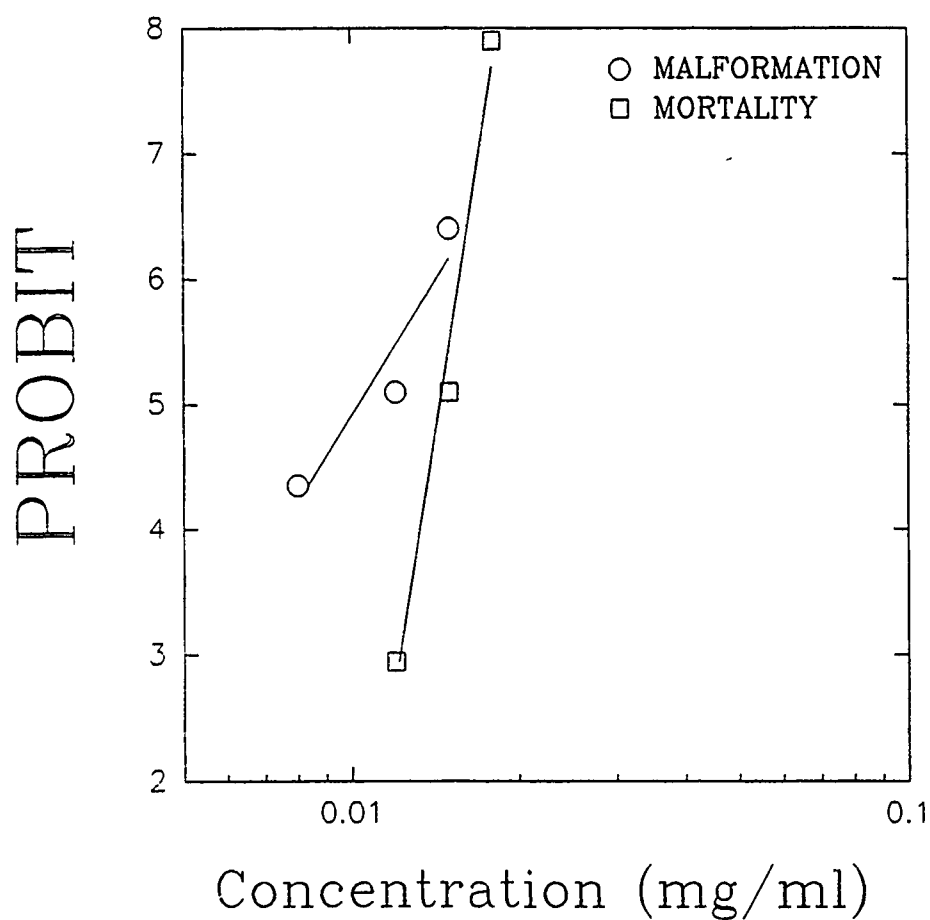


Figure 56 96 h Mortality and Malformation Dose-Response Curves for Furazolidone Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were used as well. Each point represents 50 embryos for each concentration.

FURAZOLIDONE

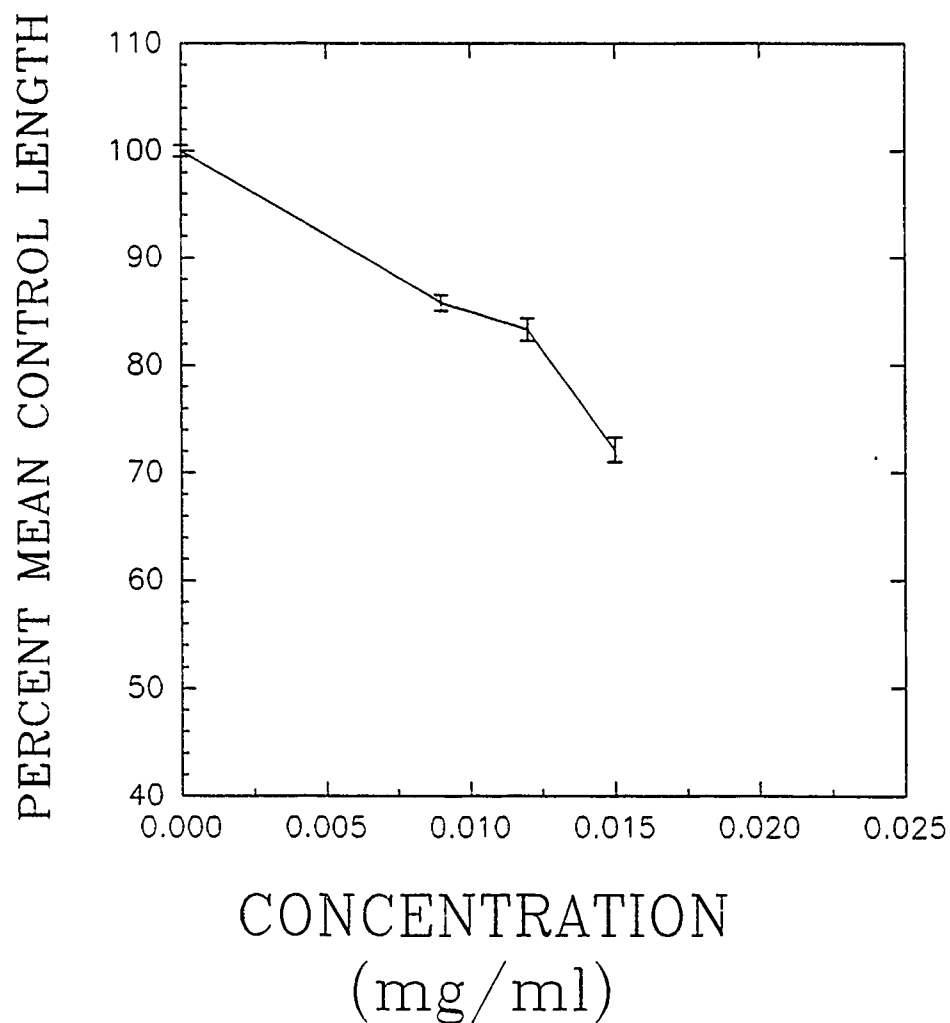


Figure 57 96 h Growth Dose-Response Curve for Furazolidone
Definitive Test #1.

FURAZOLIDONE

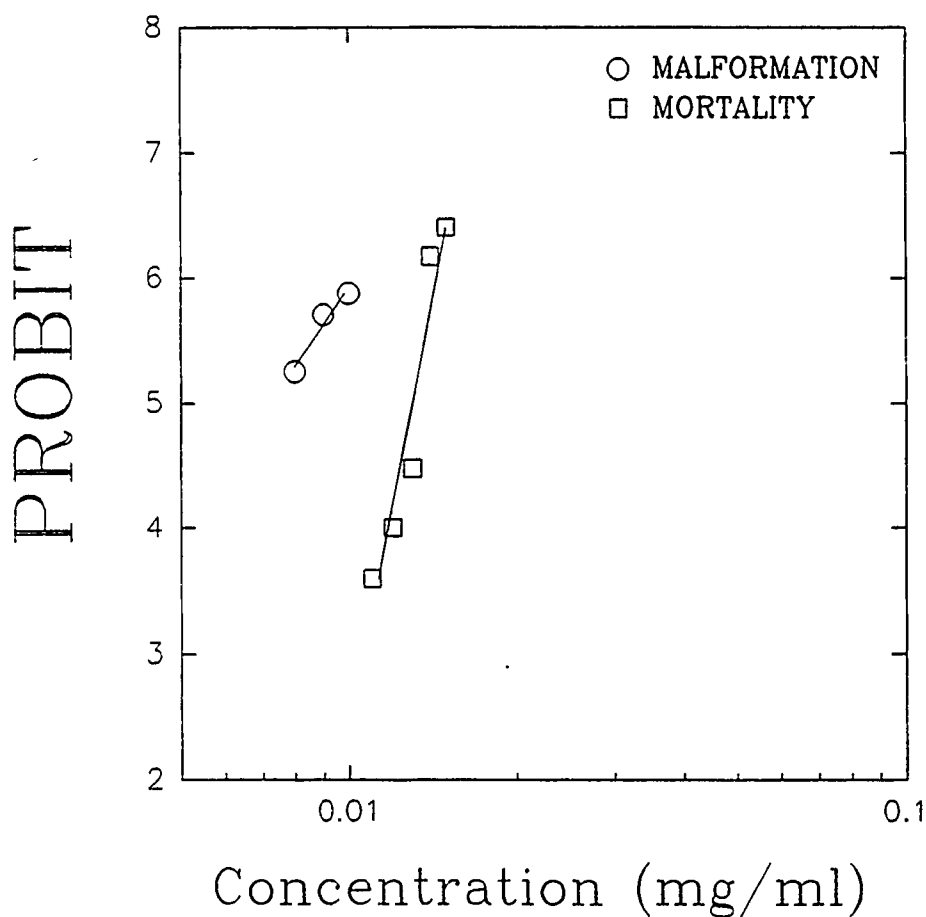


Figure 58 96 h Mortality and Malformation Dose-Response Curves for Furazolidone Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

FURAZOLIDONE

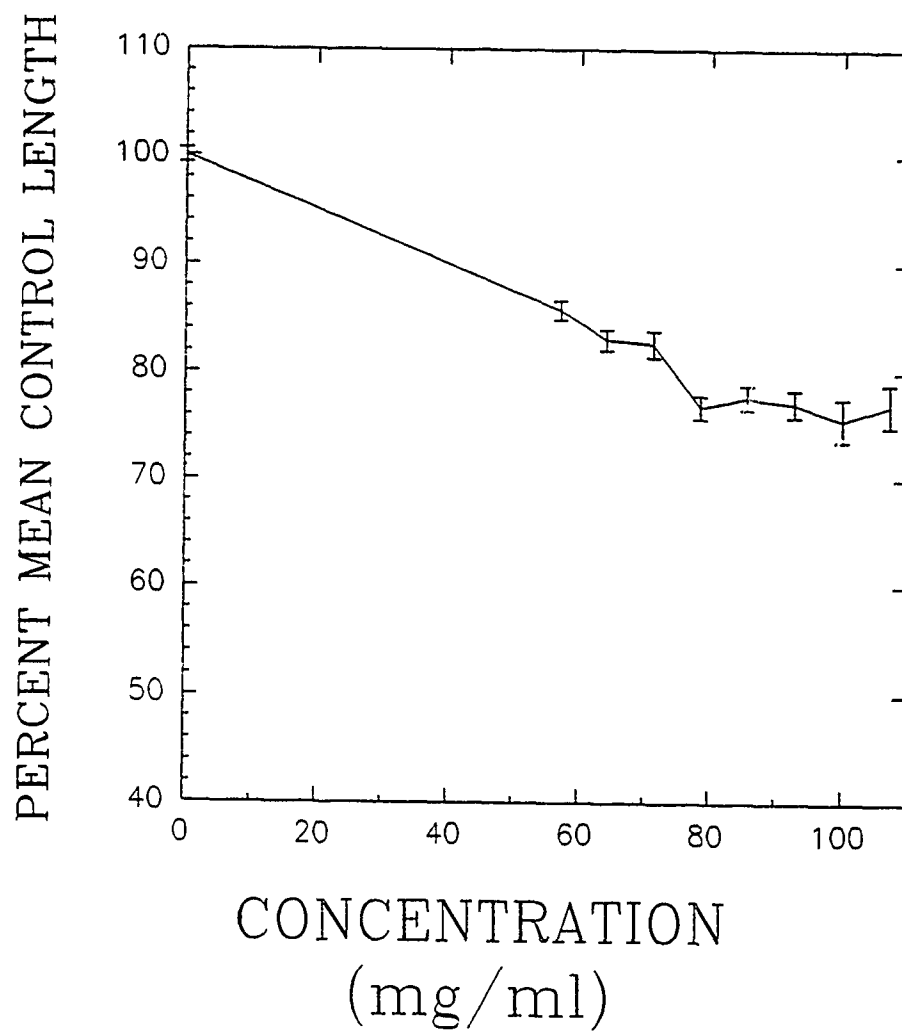


Figure 59 96 h Growth Dose-Response Curve for Furazolidone
Definitive Test #2.

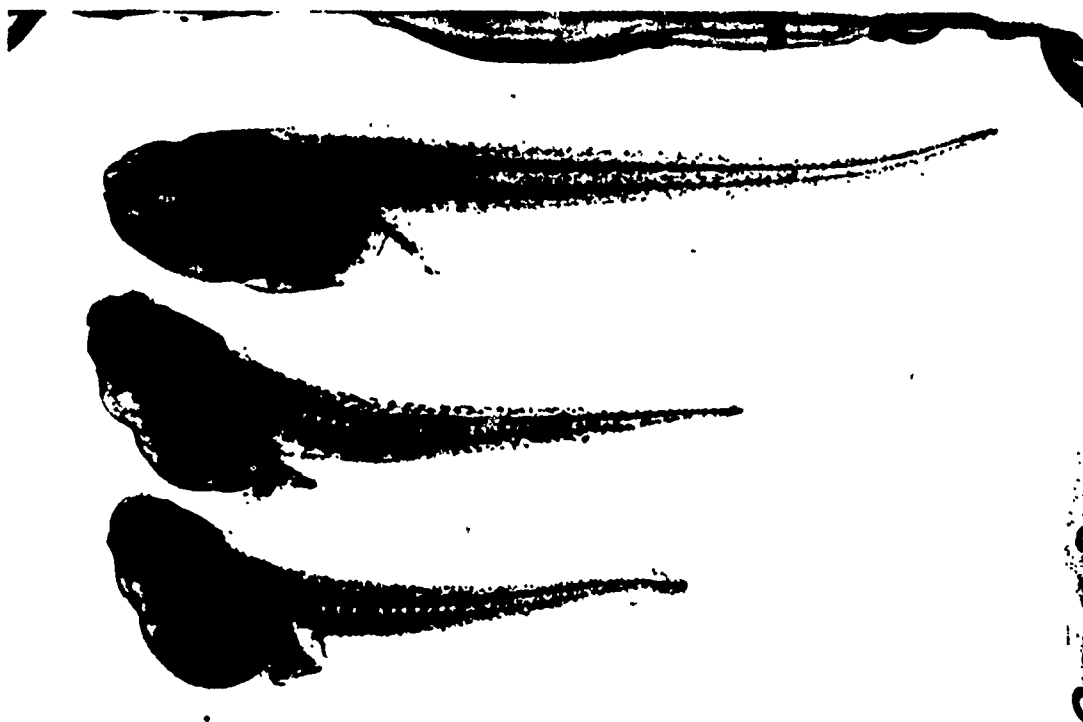


Plate 24 Effects of different concentrations of Furazolidone on Xenopus development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on face, gut and notochord. From top to bottom: control, 0.014 mg/ml, 0.015 mg/ml.

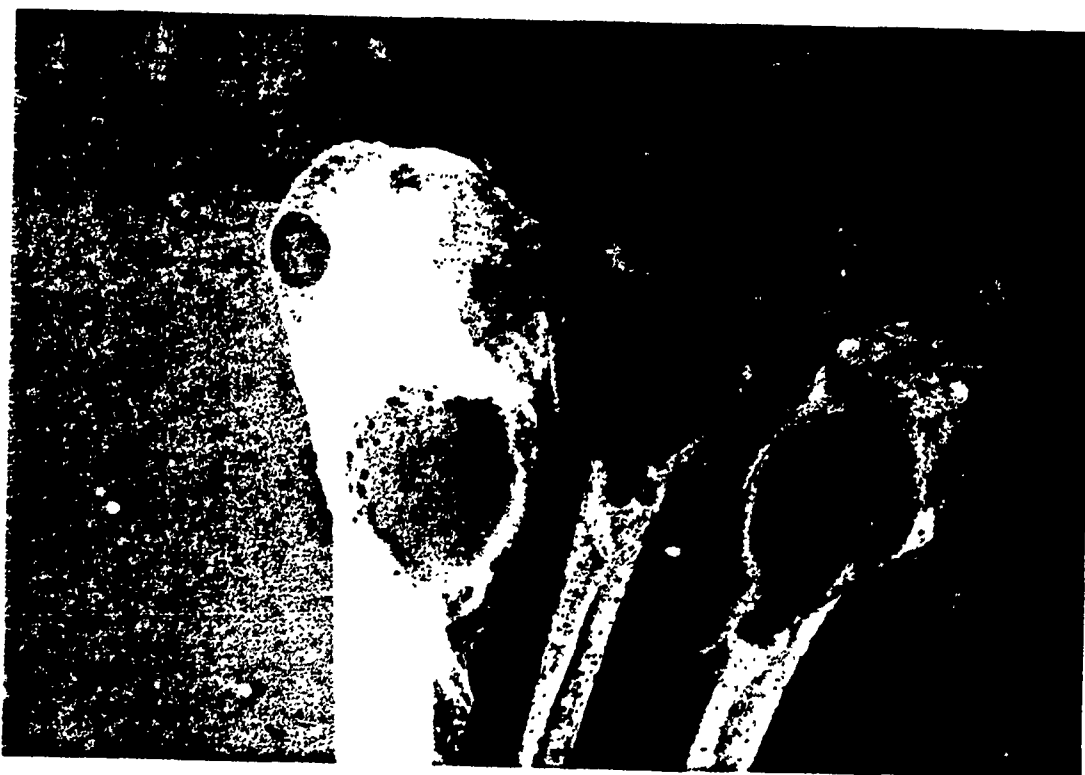


Plate 25 Ventral view of tadpoles in previous figure. From left to right: control, 0.014 mg/ml, 0.015 mg/ml.

PROCARBAZINE is considered a teratogen in the rat, mouse and rabbit. It is used as an anticancer agent in humans in chemotherapy against Hodgkin's disease, several types of lymphomas and other diseases. It is on the Flint List.

Procarbazine readily dissolves in FETAX, so no solvent is necessary. Only one good definitive test has been completed. Figure 60 shows the mortality and malformation dose-response curves for this test. The LC50 was 3.17 mg/ml and the EC50 was 1.31mg/ml. This gave a T.I. of 2.42. The most common malformations were a malformed face and blisters present along the body. Other common malformations were a loose or no gut coil and kinks in the notochord. Figure 61 represents the growth dose-response curve. The slope of the line is very steep. Strong teratogens often cause reduction in growth at very low percentages. Another test gave similar results. It cannot be considered a definitive test because an EC50 could not be established. In Figure 62 the mortality and malformation dose-response curves show an LC50 of 1.69 mg/ml. The malformation curve was figured from a transformation table to find the corresponding probit values. The most common malformations were again in the face, but no blisters were found. The notochord and gut abnormalities could also be seen. The growth dose-response curve is similar as seen in Figure 63.

We conclude that this chemical is a strong teratogen as indicated by the high T.I. and by the slope of the growth dose-response curve. This supports the results of the mammalian tests.

PROCARBAZINE

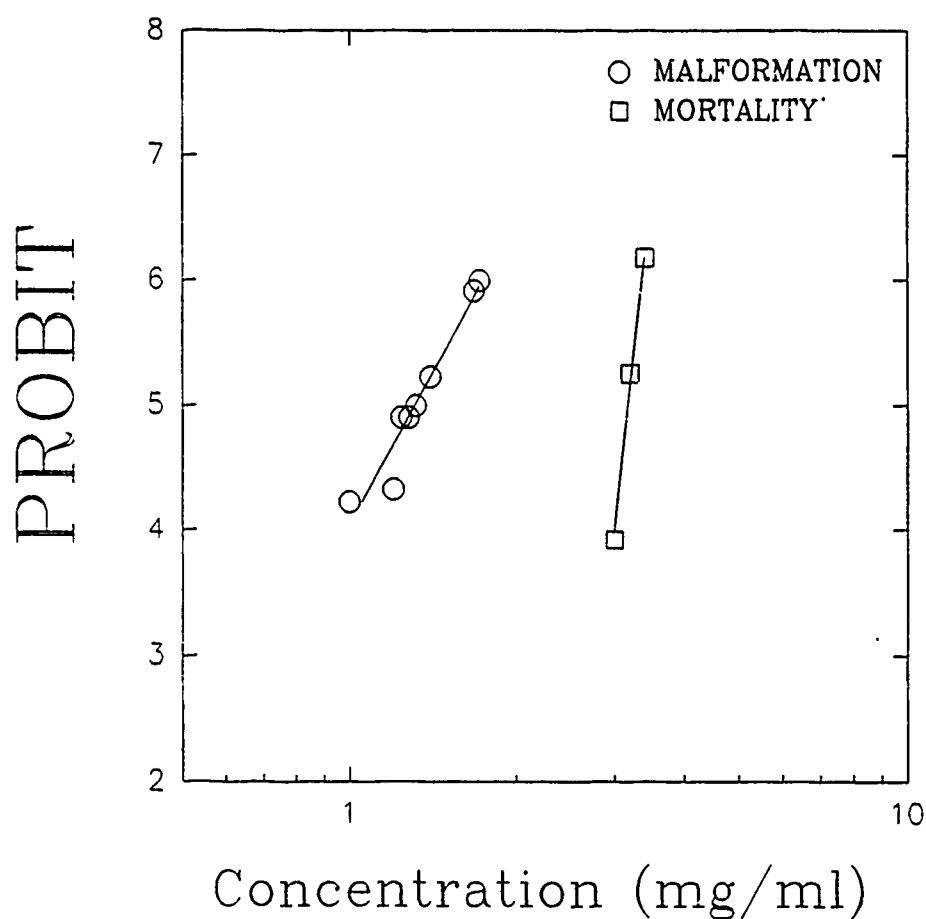


Figure 60 96 h Mortality and Malformation Dose-Response Curves for Procarbazine Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

PROCARBAZINE

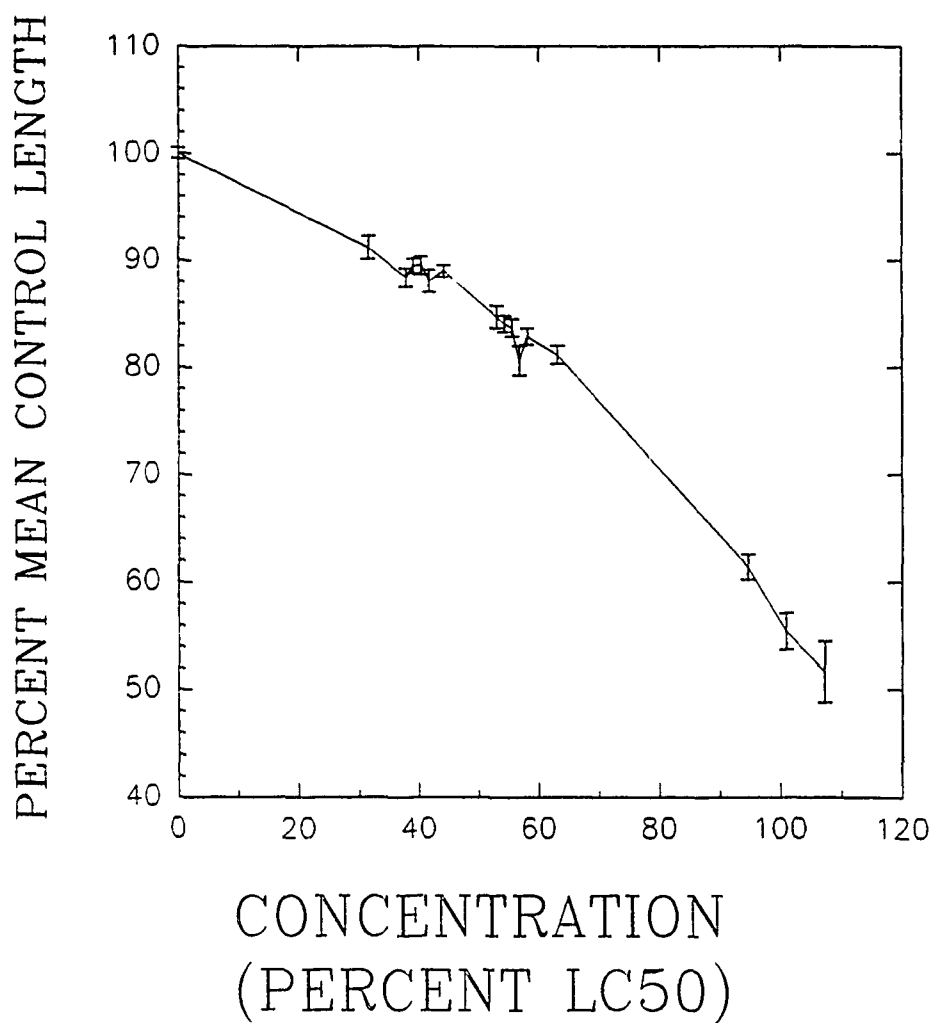


Figure 61 96 h Growth Dose-Response Curve for Procarbazine
Definitive Test #1.

PROCARBAZINE

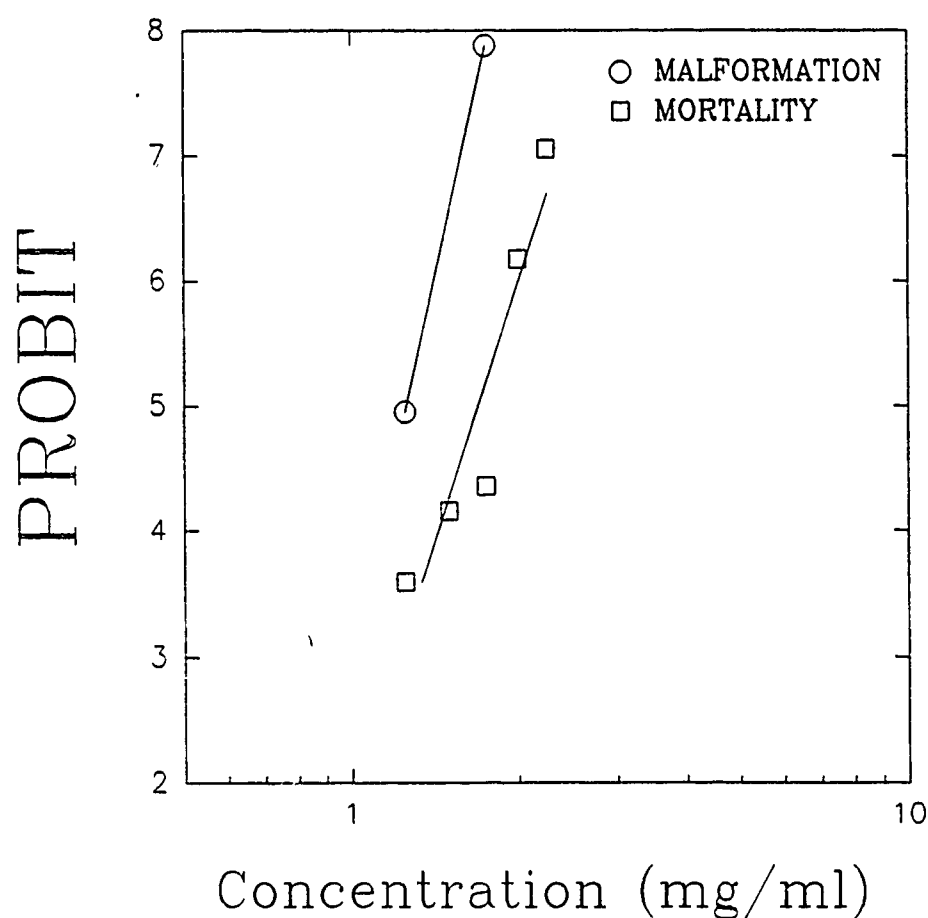


Figure 62 96 h Mortality and Malformation Dose-Response curves for Procarbazine Non-Definitive test. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

PROCARBAZINE

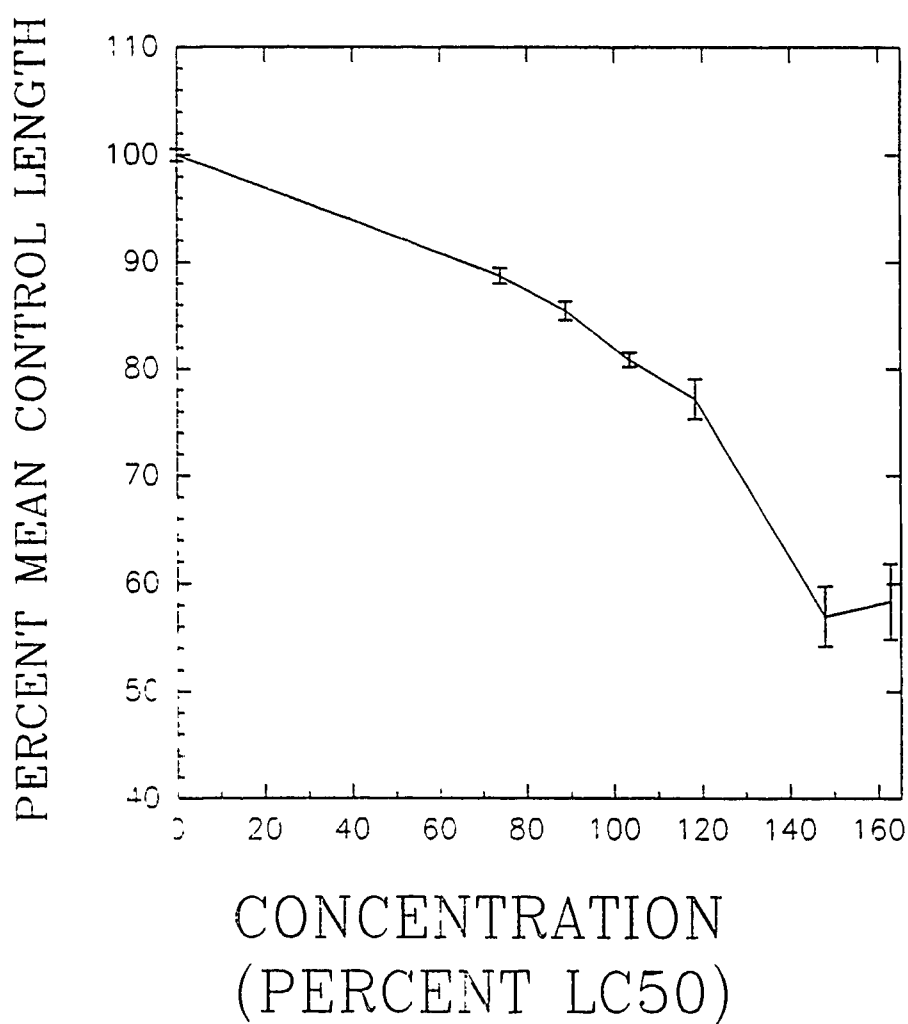


Figure 63 96 h Growth Dose-Response Curve for Procarbazine
Non-Definitive test.



Plate 26 Effects of different concentrations of Procarbazine on Xenopus development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on gut, face and notochord. From top to bottom: control, 1.28 mg/ml, 1.72 mg/ml, 2.0 mg/ml.

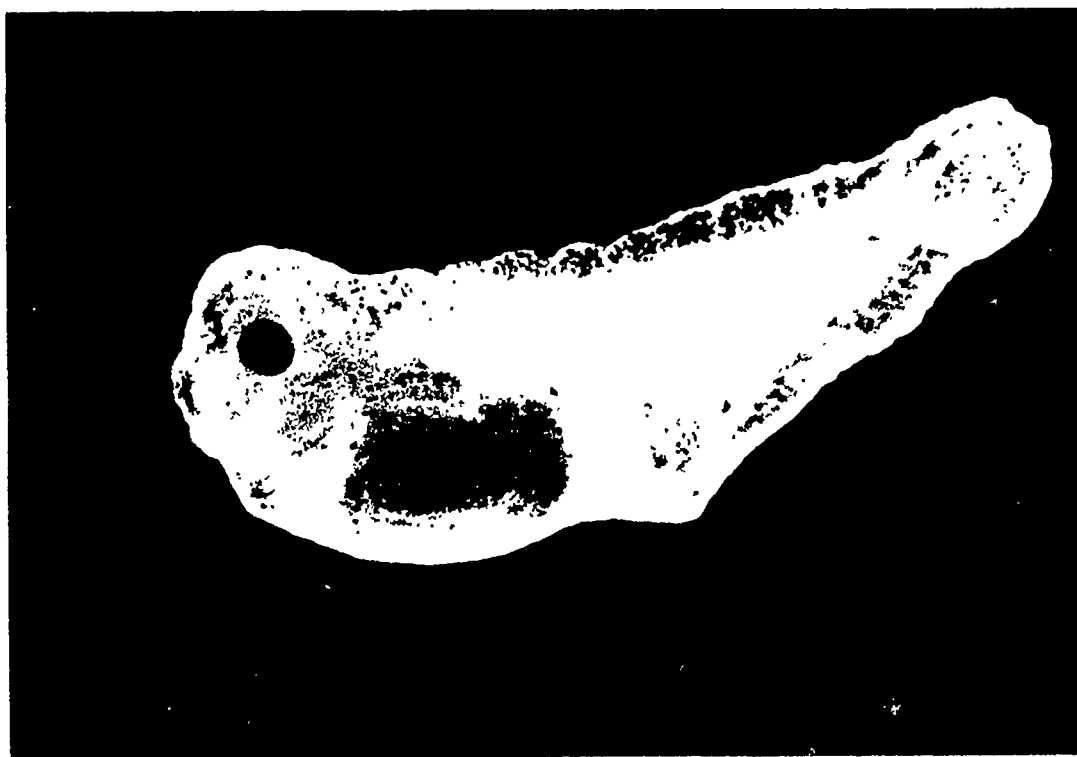


Plate 27 Effect of a high concentration of Procarbazine on Xenopus development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on face, gut and notochord. Blisters can also be seen. Embryo exposed to 3.4 mg/ml Procarbazine.

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2. SOLVENT INTERACTION

INTRODUCTION

The large number of water-insoluble chemicals requiring toxicity testing necessitates the development, validation and use of chemical cosolvents. Carrier solvents (cosolvents), such as dimethylsulfoxide (DMSO), acetone, and triethylene glycol (TG), are commonly used to solubilize hydrophobic compounds.¹ However, the use of solvents with *in vitro* bioassays may alter the developmental toxicity of test materials. Solvents interact with other compounds to change rates of reactions, membrane potentials, mutagenic activity, and many other cell processes.²⁻⁵ Using microbial assays, Stratton has shown that solvents alter the toxicity of pesticides.⁶ For this reason, solvent-compound interaction studies were performed to determine if the developmental toxicity of test materials was altered.

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), described by Dumont et al., is a 96-hr bioassay which determines relative teratogenic hazard.⁷ Several labs have evaluated compounds as well as environmental mixtures with FETAX.⁷⁻¹² An exogenous metabolic activation system has been developed and evaluated for FETAX.¹³⁻¹⁵ The purpose of these experiments was to determine whether carrier solvents interacted with the teratogens methylmercury chloride, trichloroethylene, trans-retinoic acid and 6-aminonicotinamide to affect survival, development and growth of *Xenopus* embryos.

Experimental

Chemicals

Solvents were dimethylsulfoxide (DMSO) (CAS# 67-68-5; Sigma Chemical Co., St. Louis, MO) acetone (CAS# 67-64-1; Fisher Scientific, Houston, TX), and triethylene glycol (TG) (CAS# 112-27-6; Aldrich Chemical Co., Milwaukee, WI). Teratogens were methylmercury chloride (MMC) (CAS# 115-09-3; FW 251.1; Pfaltz & Bauer Inc., Waterbury, CT), trichloroethylene (TCE) (CAS# 79-01-6; FW 131.39; Aldrich Chemical Co.), trans-retinoic acid (RA) (CAS# 302-79-4; FW 300.4; Sigma Chemical Co.) and 6-aminonicotinamide (6-AN) (CAS# 329-89-5; FW 137.1; Sigma Chemical Co.).

Assay Procedure

Animal care and breeding were performed according to Bantle et al. (1989).¹⁶ FETAX solution, a reconstituted water medium, was used as the diluent for all experiments.¹⁷ For each concentration-response test, two groups of 25 embryos each were placed in 60 X 15 mm glass Petri dishes containing a total of 10 ml of solution. Four groups of 25 embryos were exposed to FETAX solution and used as controls for each test. Because of the possible binding of methylmercury to glass, the concentration-response experiments were performed in 60 X 15 mm plastic Petri dishes containing 20 embryos and a total of 8 ml of solution. Each experiment followed standard methods of test operation and embryo evaluation.^{16,18} Stock concentrations of MMC were also determined using cold vapor method for determining mercury concentration.¹⁹

One range and three definitive experiments were performed to determine the 96-hr LC50, 96-hr EC50 (malformation), Teratogenic Index (TI) (96-hr LC50/ 96-hr EC50) and Minimum Concentration to Inhibit Growth (MCIG) for three solvents and two teratogens. The 96-hr, LC25 and LC50, and 96-hr, EC25 and EC50 (malformation) were determined using Litchfield-Wilcoxon probit analysis.²⁰ Dunnett's test was used to determine the No Observable Effect Concentrations (NOEC) for malformation and mortality. The MCIG was determined by comparing head-tail lengths between control and experimental groups using the t-test for grouped observations. These data were used in determining test concentrations for the solvent-compound interaction study. Malformations were determined using a dissecting microscope to observe deviations from normal development.²¹ Typical examples of malformations are reduced head compared with rest of embryo, reduced eye, mouth, misshapen head, eye, heart, mouth, tail fin, notocord, etc. The 96-hr embryo is transparent and most internal organs can be seen clearly. Stunting (growth inhibition) is not considered a malformation. Growth is assessed by measuring embryo head-tail lengths.

Interaction Study

Two concentrations for each solvent and test material were selected. The two solvent concentrations were the NOEC and 96-hr

EC25. The NOEC is the highest possible cosolvent concentration that can be used in FETAX. The 96-hr EC25 was chosen in order to positively identify any interactions that might be taking place that were not observable at lower concentrations. MMC and TCE concentrations were the 96-hr EC25 and 96-hr LC25. These concentrations allowed examination of effects on both malformation and mortality. All teratogens were soluble at these concentrations without the solvents. The interaction experiments contained individual treatments for all concentrations and the interaction treatments for one solvent and one teratogen per experiment. The nine treatments were: 1) FETAX solution controls, 2) EC25 of teratogen, 3) LC25 of teratogen, 4) NOEC level of solvent, 5) EC25 of solvent, 6) EC25 of teratogen & NOEC of solvent, 7) EC25 of teratogen & EC25 of solvent, 8) LC25 of teratogen & NOEC of solvent, and 9) LC25 of teratogen & EC25 of solvent. All interaction experiments contained four replicates of 25 embryos each per treatment with the exception of one MMC-DMSO interaction study which contained 22 embryos per treatment. Results of solvent-teratogen interaction studies represent three pooled experiments using three different breeding pairs. Every 24 hrs, dead embryos were removed and solutions changed. After 96 hrs of exposure, embryos were anesthetized with 3-Aminobenzoic Acid Ethyl Ester (MS-222) (CAS# E1-052-1; Sigma) and the number of malformed larvae recorded. Larvae were then killed and fixed with 3.0% (w/v) formalin and head-tail length (growth) measured.

ANOVA was used to determine differences from theoretical additive values for mortality, malformation and growth.²² Values were determined by partitioning the sum of squares for the interactions on mortality and malformation using the statistical software SYSTAT.²³ Growth interactions were determined in the same manner with the Statistical Analysis System (SAS). Effects on the TI were inferred from shifts of the mortality and malformation curves. In the case of the LC25 of the teratogens, 100% malformations were obtained without solvent addition. Therefore, only a decrease in malformation could be observed and not an increase. For these experiments, decreases in malformation were not observed for the 96-hr LC25 concentration of the teratogens.

RESULTS

PRELIMINARY EXPERIMENTS

Carrier Solvent Results

Previous work indicated that all solvents caused effects at 2.0% (v/v) concentrations. Acetone produced the highest TI value of the solvents followed by DMSO and then TG (Table 5). NOEC levels for DMSO, acetone, and TG for any endpoint were 1.0% (v/v), 0.9% (v/v), and 1.7% (v/v), respectively. The 96-hr EC25 (malformation) for DMSO, acetone and TG were 1.2% (v/v), 1.0% (v/v), and 2.0% (v/v), respectively.

Developmental Toxicity of the Teratogens

All the teratogens proved to have teratogenic potential in FETAX. The ranking of most teratogenic potential to least was 6-AN > RA > TCE > MMC (Table 5) based only on TI values. The ranking from most toxic to the least based on average 96-hr LC50 is as follows: MMC (0.4 μ M) > RA (1.2 μ M) > TCE (2.94 mM) > 6-AN (22.39 mM). The 96-hr EC25 (malformation), and 96-hr LC25 for each teratogen were as follows: Retinoic acid were 0.02 mg/L and 0.25 mg/L; 6-aminonicotinamide were 2 mg/L and 2500 mg/L; MMC were 0.015 mg/L and 0.088 mg/L; TCE were 0.002% (v/v) and 0.035% (v/v). It was not possible to determine MCIG or confidence limits for the first TCE test. However, acceptable confidence limits were obtained in all other tests. The data from these experiments was used to predict the EC25 and LC25 for the teratogens. The estimation of the EC25 for the three independent experiments for TCE ranged from 19.5% to 27.5% and the estimation of the LC25 ranged from 25.7% to 38.3% (Tables 11-13). Therefore, the estimates of EC25 and LC25 were well within acceptable limits. The 6-AN experiments (Tables 5-7) indicated that 2500 mg/L was closer to the 96-hr LC50 than the 96-hr EC50. This result could have been caused because Dawson et al. used antibiotics in the test concentrations to control bacterial growth.¹⁸ Antibiotics were not used in our experiments because possible interactions with the antibiotics. The mean MCIG for MMC was 0.038 mg/L, for TCE was 0.02% (v/v), for RA was 0.07 mg/L and for 6-AN was 100 mg/L.

INTERACTION EFFECTS CAUSED BY SOLVENTS

Control Results

Tables 2 through 13 show the pooled percentage results for all experiments. Control mortality and malformation was equal to or below 10% for all but three experiments and was never greater than

12%. Although the control malformation and mortality varied from experiment to experiment, final results were not affected because ANOVA takes into account control responses. Table 12 - Table 14 shows that mortality due to 0.088 mg/L MMC ranged from 18.0% to 41.7%. Because of this variability, values were kept discrete for each solvent-teratogen interaction. These are the percentages used to calculate the theoretical additive values. The percentages are the 12 replicate values averaged together, and the standard error is the error of these 12 means.

Methylmercury Chloride Interactions

MMC combined with DMSO caused significant ($p=0.05$) interaction only at 1.2% (v/v) DMSO and 0.088 mg/L MMC (Figure 64). Mortality was increased by 18% (6-35%; 95% confidence interval). In all cases, the percent change refers to an increase or decrease compared to the theoretical additive value. DMSO increased the mortality which should decrease the 96-hr LC50 and did not significantly increase malformation. Therefore, the 96-hr EC50(malformation) should remain the same with DMSO. This would reduce the TI of MMC. One experiment contained 22 embryos per replicate due to a shortage of embryos, and is the reason the total N per treatment is different. All experiments showed similar results.

When MMC was tested with acetone there were significant interactions observed for both mortality and malformation (Figure 64). MMC at 0.015 mg/L with 0.9% (v/v) and 1.0% (v/v) acetone increased malformation by 16.9% (10.9-23.8%; 95% confidence interval) and 28.6% (21.0-36.9%; 95% confidence interval), respectively. MMC at 0.088 mg/L with 1.0% (v/v) acetone increased the mortality by 23.7% (10.8-40%; 95% confidence interval). Because the synergistic responses for malformation and mortality were approximately the same, acetone would change the 96-hr LC50 and 96-hr EC50 (malformation) the same. The TI, therefore, should not change.

MMC combined with TG showed no significant interactions on mortality or malformation (Figure 64). TG, therefore, would not change the TI of MMC.

The TI would be expected to change for MMC only with DMSO. With acetone, the interaction is an equal increase in mortality and malformation over the expected additive value. This would increase the sensitivity of the embryos to MMC when combined with acetone. Finally, when MMC was tested with TG neither mortality nor malformation were changed. MMC growth was not changed from additive effects for any of the solvents.

Trichloroethylene Interactions

Trichloroethylene combined with DMSO had significant ($p=0.001$) additive effects on mortality (Figure 65). Mortality for 0.035% (v/v) TCE was increased over theoretical additive values for DMSO at 1.0% (v/v) and 1.2% (v/v) by 42.7% (21.7-67.7%; 95% confidence interval) and 45.3% (23.7-70.6%; 95% confidence interval), respectively. However, malformation was not changed signifi-

cantly from theoretical additive values. The increase in mortality resulted in a decreased 96-hr LC50. Because malformation was unaffected, the TI would be lowered.

TCE combined with acetone significantly ($p = 0.05$) increased mortality (Figure 65). The rate of mortality caused by TCE at 0.035% (v/v) was increased with acetone at 0.9% (v/v) and 1.0% (v/v) by 16.7% (6.4-26.7; 95% confidence interval) and 24% (13-37.5%; 95% confidence interval), respectively. The rate of TCE-induced malformation at 0.002% (v/v) was not significantly different from theoretical additive values. An increase in mortality for acetone and no change in malformation from theoretical values would again cause a reduction of the TI.

TCE combined with TG caused significant ($p = 0.05$) interaction for both mortality and malformation (Figure 65). The rate of TCE-induced mortality caused by 0.035% (v/v) TCE with 1.7% (v/v) and 2.0% (v/v) TG was increased over theoretical additive values by 15% (6.2-26.9%; 95% confidence interval) and 20.33% (9.97-33.4%; 95% confidence interval), respectively. The malformation caused by 0.002% (v/v) TCE and 2.0% (v/v) TG was significantly increased by 17.2% (10.9-24.6%; 95% confidence interval). Because both malformation and mortality for TG showed an increase of approximately the same magnitude, the TI would not be expected to change.

Trichloroethylene with DMSO and TCE with acetone caused an increase in mortality and did not change malformation significantly. TCE with TG, however, increased both mortality and malformation.

Tables 2 and 3 show the treatment effects for each of the six solvent interaction experiments. Control malformation and mortality generally were less than 8% except for two experiments (Tables 2 and 3). Table 7 revealed that 2500 mg/L 6-AN was not the LC25 but was actually closer to the LC50. The reason for these discrepancies may be attributed to an antibiotic used by Dawson et al. (1989) to control bacterial growth and in the present study no antibiotic treatments were used. Because the values for solvent and compounds often varied due to different breeding pairs, the values were kept discrete for each experiment. For example, DMSO at 1.2% (v/v) should have caused 25 percent malformation. The retinoic acid experiment (Table 6) showed 20.9% malformation for 1.2% (v/v) DMSO, and the 6-aminonicotinamide (Table 7) showed 28.3% malformation for 1.2% (v/v) DMSO.

trans-Retinoic Acid

The interaction results for RA combined with DMSO (Figure 66) showed that mortality increased significantly at $p = 0.001$. DMSO at 1% (v/v) and 1.2% (v/v) concentrations with 0.25 mg/L RA increased mortality by 34.3% and 47.3%, respectively. Because malformation of DMSO with RA was not significantly different at either concentration, the synergistic effect on mortality should cause the mortality curve to shift to the left (reducing the 96-hr LC50). DMSO addition would reduce the TI for RA.

RA in the presence of acetone (Figure 66) significantly increased malformation by 38.5% with 1% (v/v) acetone and 0.02 mg/L RA while not changing the mortality at 1% acetone and 0.25 mg/L RA. The synergistic shift of the malformation concentration-response curve to the left (reducing the 96-hr EC50) would increase the TI with acetone at the 1% (v/v) level. There were no significant effects at the 0.9% (v/v) level (NOEC) of acetone.

TG with RA had effects on both malformation and mortality (Figure 66). Malformation with 2.0% (v/v) TG and 0.02 mg/L RA reduced the additive value by 7% at $p = 0.059$. Mortality rates of 1.7% (v/v) TG and 2.0% (v/v) TG with 0.25 mg/L RA increased the additive values by 12.3% and 23% respectively. The antagonistic shift of the malformation curve to the right (increasing the 96-hr EC50) and the synergistic shift of the mortality curve to the left (decreasing 96-hr LC50) would produce a reduced TI.

6-Aminonicotinamide

DMSO combined with 6-AN synergistically increased mortality rates at both levels of the solvent at $p = 0.001$ (Figure 67). The increase in mortality for 1% (v/v) DMSO and 1.2% (v/v) with 2500 mg/L 6-AN was 49.3% and 45.7%, respectively. The mortality curve would shift to the left, decreasing the 96-hr LC50 and reducing the TI.

Acetone tested with 6-AN had significant effects at $p \leq 0.05$ for both malformation and mortality (Figure 67). The malformation decreased with 0.9% (v/v) acetone and 2 mg/L 6-AN by 6.8%. However, the 1.0% (v/v) acetone with 2 mg/L 6-AN increased malformation by only 1.2%. The mortality caused by acetone with 6-AN increased for 0.9% (v/v) acetone and 1% (v/v) acetone with 2500 mg/L 6-AN by 39% and 38% respectively. Because the synergistic increase in mortality (reducing the 96-hr LC50) outweighed the antagonistic effects on malformation, the TI should be reduced.

TG in the presence of 6-AN significantly increased both malformation and mortality at $p \leq 0.05$ (Figure 67). TG at 1.7% (v/v) and 2% (v/v) combined with 2 mg/L 6-AN increased malformation by 34.5% and 14.2%, respectively. Mortality increased for 1.7% (v/v) TG and 2.0% (v/v) with 2500 mg/L 6-AN by 12.7% and 11.3%, respectively. Both mortality and malformation were both increased by approximately (reducing both the 96-hr LC50 and 96-hr EC50) the same amount, therefore, the TI should not change appreciably.

DISCUSSION

The Teratogenic Index (TI) is the ratio of the 96-hr LC50/96-hr EC50 (malformation) and represents the separation between the mortality and malformation curves. It would be expected that for compounds to induce malformations without causing mortality that the 96-hr EC50 (malformation) should be less than the 96-hr LC50. This is not, however, the only way to classify compounds because the number of malformations do not give information on the types or severity of these malformations. The separation between the two curves is considered to be sufficient to pose significant teratogenic hazard when the $TI > 1.5$.^{16,18} If a synergistic or antagonistic response altered a TI, false conclusions regarding teratogenic hazard could result.²⁴

MMC is known to cause cleft palate in mice.²⁵ MMC is also known to affect development of several fetal systems.²⁶⁻²⁸ TCE is known to induce strand breaks in DNA in the rat and mouse liver in vivo and to be weakly mutagenic.²⁹⁻³⁰ TCE causes abnormal development of chick embryos.³¹⁻³² However, TCE does not show strong teratogenic effects in the rat inhalation studies but did produce skeletal anomalies.³³ Route of exposure is important when comparing relative toxicity, teratogenicity. TCE seems to show wide species variation in response. These sources support that MMC and, perhaps, TCE should be teratogenic in FETAX. Mammalian literature indicated that 6-AN caused congenital malformations.³⁴⁻³⁵ RA has also been shown to cause embryo defects, such as malformed limbs.³⁶⁻³⁷

The interaction results show that the carrier solvents do interact with teratogens. Of the three indicators measured, mortality and malformation were potentiated, while growth was not affected. Typically, growth and malformation are the more sensitive endpoints. However, more interactions were discovered with mortality. This shows that a change in one endpoint does not necessarily result in a change in the others. This study also shows that the choice of carrier solvents is critical because the different carrier solvents caused different interactions. For example, only acetone caused effects at the NOEC with MMC. However, all solvents caused effects when combined with TCE at the NOEC. While TG had no observable effects with MMC, both malformation and mortality were significantly changed with TCE.

Malformations caused by all interaction treatments did not produce new or different types of malformation. All malformations were the same type as seen with individual control treatments for each teratogen and solvent. Differences were in the magnitude of the response and the number of malformations. Acetone has previously been shown to change mutagenic potential of N-Methyl-N-nitrosourea and to interact on membrane integrity.^{5,38} DMSO has been shown to interact with secalonic acid D to alter teratogenicity in mice.³⁹ These papers support the results of this study where interactions were found between MMC and TCE, and the three solvents.

Carrier solvents, although sometimes necessary, need to be used with caution at the lowest solvent concentrations possible. If possible, several different carrier solvents should be used separately to determine

if the results are consistent.⁴⁰ Fewer effects were seen at the NOEC than at the 96-hr EC50 for the solvents. Also interesting, is the finding that MMC was the least teratogenic and had the fewest interactions, while 6-AN, with the highest TI, had the most interactions.

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Table 5

The 96-hr LC50, 96-hr EC50 (malformation), Teratogenic Index (TI)^a, and Minimum Concentration to Inhibit Growth (MCIG) for Dimethylsulfoxide (DMSO), Acetone, Triethylene glycol (TG), Retinoic Acid (RA), 6-aminonicotinamide (6-AN), Methylmercury Chloride (MMC) and Trichloroethylene (TCE).

| Compound | Trial | 96-hr LC50 | 96hr EC50 | TI | MCIG |
|----------------------|-------|-------------------------------|---------------------|------|-------|
| DMSO ^b | 1 | 1.81(1.75-1.87 ^c) | 1.4 (1.32-1.48) | 1.3 | 1.3 |
| | 2 | 1.77(1.61-1.95) | 1.29(1.25-1.33) | 1.4 | 1.7 |
| | 3 | 1.86(1.4-2.3) | 1.24(0.83-1.8) | 1.5 | 1.5 |
| Acetone ^b | 1 | 2.16(2.07-2.25) | 1.4 (1.29-1.43) | 1.6 | 1.8 |
| | 2 | 2.49(2.10-2.95) | 1.4 (1.04-1.36) | 1.8 | 1.5 |
| | 3 | 1.92(1.90-2.14) | 1.06(0.91-1.17) | 1.83 | 1.0 |
| TG ^b | 1 | 2.4 (2.02-2.85) | 2.0 (2.01-2.13) | 1.2 | 1.8 |
| | 2 | 2.75(2.70-2.82) | 2.4 (2.37-2.45) | 1.1 | 1.8 |
| | 3 | 2.19(2.19-2.32) | 2.05(1.99-2.11) | 1.07 | 1.7 |
| RA ^d | 1 | 0.25(0.22-0.28) | 0.024(0.018-0.031) | 10.4 | 0.06 |
| | 2 | 0.50(0.46-0.61) | 0.044(0.032-0.060) | 11.4 | 0.08 |
| 6-AN ^{d,e} | 1 | 3190(3000 3400) | 5.3(2.5-7.5) | 602 | 100 |
| | 2 | 2950(2800-3100) | 5.7(5.3-6.2) | 518 | NA |
| MMC ^d | 1 | 0.083(0.080-0.087) | 0.024(0.021-0.028) | 3.4 | 0.036 |
| | 2 | 0.094(0.088-0.100) | 0.025(0.018-0.034) | 3.7 | 0.04 |
| TCE ^{b,f} | 1 | 0.024(NA) | 0.0048(0.002-0.011) | 5 | NA |
| | 2 | 0.029(0.026-0.032) | 0.0023(0.001-0.004) | 12.6 | 0.02 |

^a TI = 96-hr LC50/96-hr EC50 (malformation).

^b Concentrations expressed as % (v/v).

^c 95% confidence limits.

^d Concentrations expressed as mg/L.

^e Data from Dawson et al.¹⁸.

^f Density = 1.462.

NA = Not Available.

Table 6

Effects of Dimethyl Sulfoxide (DMSO), Retinoic Acid (RA) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 11 | (3.7 ± 1.43 ^b) | 12 | (4.2 ± 0.77) | 9.61 ± 0.064 |
| DMSO RA | | | | | |
| % (v/v) (mg/L) | | | | | |
| 1.0 | 11 | (3.7 ± 0.77) | 29 | (10.1 ± 1.53) | 9.44 ± 0.092 |
| 1.2 | 22 | (7.3 ± 1.76) | 58 | (20.9 ± 0.94) | 9.17 ± 0.064 |
| 0.02 | 18 | (6.0 ± 1.15) | 65 | (23.0 ± 3.58) | 9.58 ± 0.081 |
| 0.25 | 64 | (21.3 ± 6.86) | 236 | (100.0) | 7.06 ± 0.381 |
| 1.0 0.02 | 13 | (4.3 ± 1.25) | 104 | (36.3 ± 6.06) | 9.15 ± 0.098 |
| 1.2 0.02 | 15 | (5.0 ± 1.31) | 111 | (38.9 ± 3.61) | 9.10 ± 0.069 |
| 1.0 0.25 | 167 | (55.7 ± 7.95) | 133 | (100.0) | 6.55 ± 0.305 |
| 1.2 0.25 | 217 | (72.3 ± 9.84) | 83 | (100.0) | 6.25 ± 0.460 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 7

Effects of Acetone, Retinoic Acid (RA) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 6 | (2.0 ± 0.78 ^b) | 14 | (4.7 ± 1.54) | 9.54 ± 0.046 |
| Acetone RA | | | | | |
| %(v/v) (mg/L) | | | | | |
| 0.9 | 9 | (3.0 ± 1.22) | 31 | (10.6 ± 1.67) | 9.04 ± 0.061 |
| 1.0 | 13 | (4.3 ± 1.25) | 65 | (22.7 ± 1.12) | 8.94 ± 0.087 |
| 0.02 | 12 | (4.0 ± 1.21) | 93 | (32.2 ± 5.49) | 9.17 ± 0.087 |
| 0.25 | 87 | (29.0 ± 7.45) | 213 | (100.0) | 6.33 ± 0.352 |
| 0.9 0.02 | 17 | (5.7 ± 1.04) | 145 | (51.1 ± 5.18) | 8.89 ± 0.066 |
| 1.0 0.02 | 18 | (6.0 ± 2.25) | 251 | (89.2 ± 4.94) | 8.61 ± 0.098 |
| 0.9 0.25 | 132 | (44.0 ± 9.86) | 168 | (100.0) | 6.20 ± 0.211 |
| 1.0 0.25 | 120 | (40.0 ± 11.79) | 180 | (100.0) | 6.11 ± 0.140 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 8

Effects of Triethylene Glycol (TG), Retinoic Acid (RA) and their Interactions on
Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|---------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 23 | (7.7 ± 2.9 ^b) | 22 | (7.9 ± 0.67) | 9.36 ± 0.061 |
| TG RA | | | | | |
| %(v/v) (mg/L) | | | | | |
| 1.7 | 35 | (11.7 ± 2.53) | 58 | (22.1 ± 2.68) | 8.28 ± 0.144 |
| 2.0 | 27 | (9.0 ± 2.04) | 94 | (34.5 ± 2.76) | 8.31 ± 0.234 |
| 0.02 | 20 | (6.7 ± 2.22) | 68 | (24.5 ± 1.62) | 9.17 ± 0.061 |
| 0.25 | 54 | (18.0 ± 4.89) | 246 | (100.0) | 7.32 ± 0.237 |
| 1.7 0.02 | 26 | (8.7 ± 2.78) | 100 | (36.4 ± 3.72) | 8.43 ± 0.199 |
| 2.0 0.02 | 25 | (8.3 ± 2.78) | 131 | (47.7 ± 4.76) | 8.32 ± 0.210 |
| 1.7 0.25 | 115 | (38.3 ± 9.99) | 185 | (100.0) | 6.20 ± 0.390 |
| 2.0 0.25 | 139 | (46.3 ± 11.11) | 161 | (100.0) | 6.48 ± 0.498 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 9

Effects of Dimethyl Sulfoxide (DMSO), 6-Aminonicotinamide (6-AN) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 12 | (4.0 ± 1.39 ^b) | 26 | (8.9 ± 2.10) | 9.51 ± 0.115 |
| DMSO 6-AN | | | | | |
| % (v/v) (mg/L) | | | | | |
| 1.0 | 14 | (4.7 ± 1.69) | 43 | (15.0 ± 1.70) | 9.55 ± 0.124 |
| 1.2 | 25 | (8.3 ± 2.97) | 78 | (28.3 ± 3.11) | 9.60 ± 0.144 |
| 2.0 | 12 | (4.0 ± 1.30) | 71 | (24.8 ± 1.80) | 9.77 ± 0.064 |
| 2500 | 150 | (50.0 ± 5.34) | 150 | (100.0) | 6.70 ± 0.063 |
| 1.0 2.0 | 18 | (6.0 ± 1.94) | 94 | (33.4 ± 4.41) | 9.71 ± 0.167 |
| 1.2 2.0 | 19 | (6.3 ± 2.43) | 132 | (46.9 ± 6.84) | 9.56 ± 0.124 |
| 1.0 2500 | 300 | (100.0) | | | |
| 1.2 2500 | 300 | (100.0) | | | |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 10

Effects of Acetone, 6-Aminonicotinamide (6-AN) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|---------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 7 | (2.3 ± 1.25) | 15 | (5.1 ± 0.74) | 9.72 ± 0.084 |
| Acetone 6-AN | | | | | |
| %(v/v) (mg/L) | | | | | |
| 0.9 | 11 | (3.7 ± 1.04) | 43 | (15.0 ± 1.26) | 9.43 ± 0.072 |
| 1.0 | 20 | (6.7 ± 2.16) | 74 | (26.6 ± 1.61) | 9.17 ± 0.061 |
| 2.0 | 10 | (3.3 ± 1.46) | 70 | (24.2 ± 1.23) | 9.76 ± 0.075 |
| 2500 | 167 | (55.7 ± 8.76) | 133 | (100.0) | 6.06 ± 0.182 |
| 0.9 2.0 | 25 | (8.3 ± 2.89) | 75 | (27.2 ± 1.06) | 9.29 ± 0.098 |
| 1.0 2.0 | 11 | (3.7 ± 1.04) | 135 | (46.8 ± 2.87) | 9.10 ± 0.058 |
| 0.9 2500 | 288 | (96.0 ± 1.21) | 12 | (100.0) | 6.08 ± 0.084 |
| 1.0 2500 | 294 | (98.0 ± 1.15) | 6 | (100.0) | 5.84 ± 0.055 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 11

Effects of Triethylene Glycol (TG), 6-Aminonicotinamide (6-AN) and their Interactions
on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|-----------------------------------|-----------|---------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control TG 6-AN | 29 | (9.7 ± 2.97) | 17 | (6.5 ± 1.45) | 9.44 ± 0.136 |
| % (v/v) (mg/L) | | | | | |
| 1.7% | 52 | (17.3 ± 6.35) | 40 | (16.0 ± 2.42) | 9.00 ± 0.156 |
| 2.0% | 57 | (19.0 ± 6.44) | 113 | (46.6 ± 4.11) | 8.60 ± 0.202 |
| 2.0 | 26 | (8.7 ± 3.18) | 57 | (20.9 ± 1.36) | 9.50 ± 0.181 |
| 2500 | 209 | (69.7 ± 9.98) | 91 | (100.0) | 6.69 ± 0.147 |
| 1.7% 2.0 | 64 | (21.3 ± 6.27) | 168 | (71.4 ± 7.47) | 8.60 ± 0.240 |
| 2.0% 2.0 | 33 | (11.0 ± 5.10) | 218 | (81.6 ± 4.14) | 8.40 ± 0.240 |
| 1.7% 2500 | 299 | (99.7 ± 0.33) | 1 | (100.0) | 7.74 |
| 2.0% 2500 | 300 | (100.0) | | | |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 12

Effects of Dimethyl Sulfoxide (DMSO), Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 18 | (6.3 ± 2.72 ^b) | 18 | (7.0 ± 1.42) | 9.34 ± 0.069 |
| DMSO MMC | | | | | |
| % (v/v) (mg/L) | | | | | |
| 1.0 | 23 | (8.3 ± 1.94) | 24 | (9.4 ± 1.34) | 9.16 ± 0.069 |
| 1.2 | 27 | (9.6 ± 2.43) | 75 | (29.8 ± 3.92) | 8.95 ± 0.072 |
| 0.015 | 19 | (6.7 ± 2.71) | 50 | (19.3 ± 1.65) | 9.22 ± 0.086 |
| 0.088 | 117 | (41.7 ± 8.26) | 163 | (100) | 7.55 ± 0.129 |
| 1.0 0.015 | 22 | (7.7 ± 2.33) | 65 | (25.0 ± 2.34) | 9.21 ± 0.086 |
| 1.2 0.015 | 70 | (25.0 ± 7.75) | 109 | (51.8 ± 9.18) | 8.85 ± 0.090 |
| 1.0 0.088 | 123 | (44.0 ± 9.62) | 157 | (100) | 7.88 ± 0.213 |
| 1.2 0.088 | 176 | (63.0 ± 10.33) | 104 | (100) | 7.68 ± 0.253 |

^a N for all treatments equaled 288 embryos from three separate experiments. Two experiments contained 25 embryos per dish (8 dishes), and one experiment contained 22 embryos per dish (4 dishes).

^b Standard Error of Mean.

Table 13

Effects of Acetone, Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 22 | (7.3 ± 2.08 ^b) | 33 | (11.8 ± 2.01) | 9.47 ± 0.069 |
| Acetone MMC | | | | | |
| % (v/v) (mg/L) | | | | | |
| 0.9 | 23 | (7.7 ± 2.89) | 56 | (20.3 ± 1.55) | 8.97 ± 0.064 |
| 1.0 | 16 | (5.3 ± 1.50) | 100 | (35.2 ± 1.95) | 8.78 ± 0.075 |
| 0.015 | 18 | (6.0 ± 2.44) | 63 | (22.2 ± 0.97) | 9.41 ± 0.130 |
| 0.088 | 54 | (18.0 ± 4.45) | 246 | (100) | 8.85 ± 0.127 |
| 0.9 0.015 | 21 | (7.0 ± 2.04) | 133 | (47.6 ± 4.17) | 8.95 ± 0.098 |
| 1.0 0.015 | 32 | (10.7 ± 2.76) | 199 | (74.2 ± 5.59) | 8.70 ± 0.104 |
| 0.9 0.088 | 89 | (29.7 ± 7.65) | 211 | (100) | 7.81 ± 0.135 |
| 1.0 0.088 | 119 | (39.7 ± 10.16) | 181 | (100) | 7.71 ± 0.107 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 14

Effects of Triethylene Glycol(TG), Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|-----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 30 | (10.0 ± 2.00 ^b) | 25 | (9.4 ± 1.47) | 9.27 ± 0.084 |
| TG MMC | | | | | |
| %(v/v) (mg/L) | | | | | |
| 1.7 | 15 | (5.0 ± 0.87) | 35 | (12.3 ± 1.41) | 9.14 ± 0.087 |
| 2.0 | 21 | (7.0 ± 2.04) | 103 | (36.8 ± 3.48) | 8.88 ± 0.087 |
| 0.015 | 24 | (8.0 ± 2.46) | 59 | (21.5 ± 0.99) | 8.99 ± 0.092 |
| 0.088 | 81 | (27.0 ± 10.00) | 219 | (100) | 8.00 ± 0.187 |
| 1.7 0.015 | 17 | (5.7 ± 1.59) | 93 | (32.7 ± 2.80) | 9.33 ± 0.066 |
| 2.0 0.015 | 19 | (6.3 ± 3.21) | 160 | (57.1 ± 5.80) | 9.19 ± 0.118 |
| 1.7 0.088 | 60 | (20.0 ± 6.73) | 240 | (100) | 8.06 ± 0.269 |
| 2.0 0.088 | 51 | (17.0 ± 4.15) | 249 | (100) | 8.07 ± 0.199 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 15

Effects of Dimethyl Sulfoxide(DMSO), Trichloroethylene (TCE) and their Interactions
on Mortality, Malformation and Growth.

| Treatment ^a | | Mortality | | Malformation | | Mean Length |
|------------------------|-------|-----------|----------------------------|--------------|---------------|--------------|
| % (v/v) | | No. | (%) | No. | (%) | (mm) |
| FETAX soln. Control | | 22 | (7.3 ± 3.07 ^b) | 25 | (9.0 ± 1.72) | 9.35 ± 0.101 |
| DMSO | TCE | | | | | |
| 1.0 | | 18 | (6.0 ± 2.63) | 31 | (11.0 ± 1.35) | 9.14 ± 0.072 |
| 1.2 | | 31 | (10.3 ± 4.07) | 77 | (28.8 ± 2.42) | 8.92 ± 0.124 |
| | 0.002 | 21 | (7.0 ± 6.29) | 58 | (20.9 ± 1.12) | 9.40 ± 0.118 |
| | 0.035 | 115 | (38.3 ± 10.69) | 185 | (100) | 7.73 ± 0.115 |
| 1.0 | 0.002 | 16 | (5.3 ± 1.80) | 81 | (28.7 ± 2.04) | 9.14 ± 0.089 |
| 1.2 | 0.002 | 42 | (14.0 ± 4.13) | 118 | (45.9 ± 1.78) | 8.92 ± 0.109 |
| 1.0 | 0.035 | 239 | (79.7 ± 9.24) | 61 | (100) | 7.55 ± 0.219 |
| 1.2 | 0.035 | 260 | (86.7 ± 6.44) | 40 | (100) | 7.08 ± 0.112 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Table 16

Effects of Acetone, Trichloroethylene (TCE) and their interactions on Mortality,
Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|----------------------------|--------------|---------------|--------------|
| % (v/v) | No. | (%) | No. | (%) | (mm) |
| FETAX soln. Control | 17 | (5.7 ± 2.00 ^b) | 15 | (5.15 ± 1.44) | 9.36 ± 0.069 |
| Acetone TCE | | | | | |
| 0.9 | 37 | (12.3 ± 4.52) | 47 | (17.9 ± 1.49) | 9.01 ± 0.066 |
| 1.0 | 19 | (6.3 ± 2.12) | 124 | (44.3 ± 4.13) | 8.91 ± 0.095 |
| 0.002 | 11 | (3.7 ± 1.51) | 56 | (19.5 ± 1.26) | 9.31 ± 0.072 |
| 0.035 | 77 | (25.7 ± 7.84) | 223 | (100) | 8.21 ± 0.179 |
| 0.9 0.002 | 34 | (11.3 ± 3.30) | 111 | (41.7 ± 2.93) | 9.02 ± 0.101 |
| 1.0 0.002 | 21 | (7.0 ± 3.94) | 175 | (62.7 ± 4.41) | 8.88 ± 0.081 |
| 0.9 0.035 | 147 | (49.0 ± 7.61) | 153 | (100) | 7.69 ± 0.133 |
| 1.0 0.035 | 151 | (50.3 ± 9.33) | 149 | (100) | 7.76 ± 0.268 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Table 17

Effects of Triethylene Glycol (TG), Trichloroethylene (TCE) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|-----------------------------|--------------|---------------|--------------|
| % (v/v) | No. | (%) | No. | (%) | (mm) |
| FETAX soln. | | | | | |
| Control | 32 | (10.7 ± 2.80 ^b) | 29 | (10.8 ± 2.35) | 9.29 ± 0.127 |
| TG TCE | | | | | |
| 1.7 | 18 | (6.0 ± 1.87) | 63 | (22.2 ± 2.31) | 9.13 ± 0.121 |
| 2.0 | 23 | (7.7 ± 2.17) | 101 | (36.4 ± 2.41) | 8.76 ± 0.165 |
| 0.002 | 32 | (10.7 ± 2.33) | 74 | (27.5 ± 2.70) | 9.24 ± 0.129 |
| 0.035 | 99 | (33.0 ± 9.57) | 201 | (100) | 8.03 ± 0.292 |
| 1.7 0.002 | 25 | (8.3 ± 2.28) | 132 | (48.1 ± 7.05) | 8.91 ± 0.150 |
| 2.0 0.002 | 27 | (9.0 ± 2.15) | 192 | (70.3 ± 6.97) | 8.60 ± 0.182 |
| 1.7 0.035 | 130 | (43.3 ± 10.92) | 170 | (100) | 7.62 ± 0.354 |
| 2.0 0.035 | 151 | (50.3 ± 11.01) | 149 | (100) | 7.39 ± 0.387 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Figure 64.

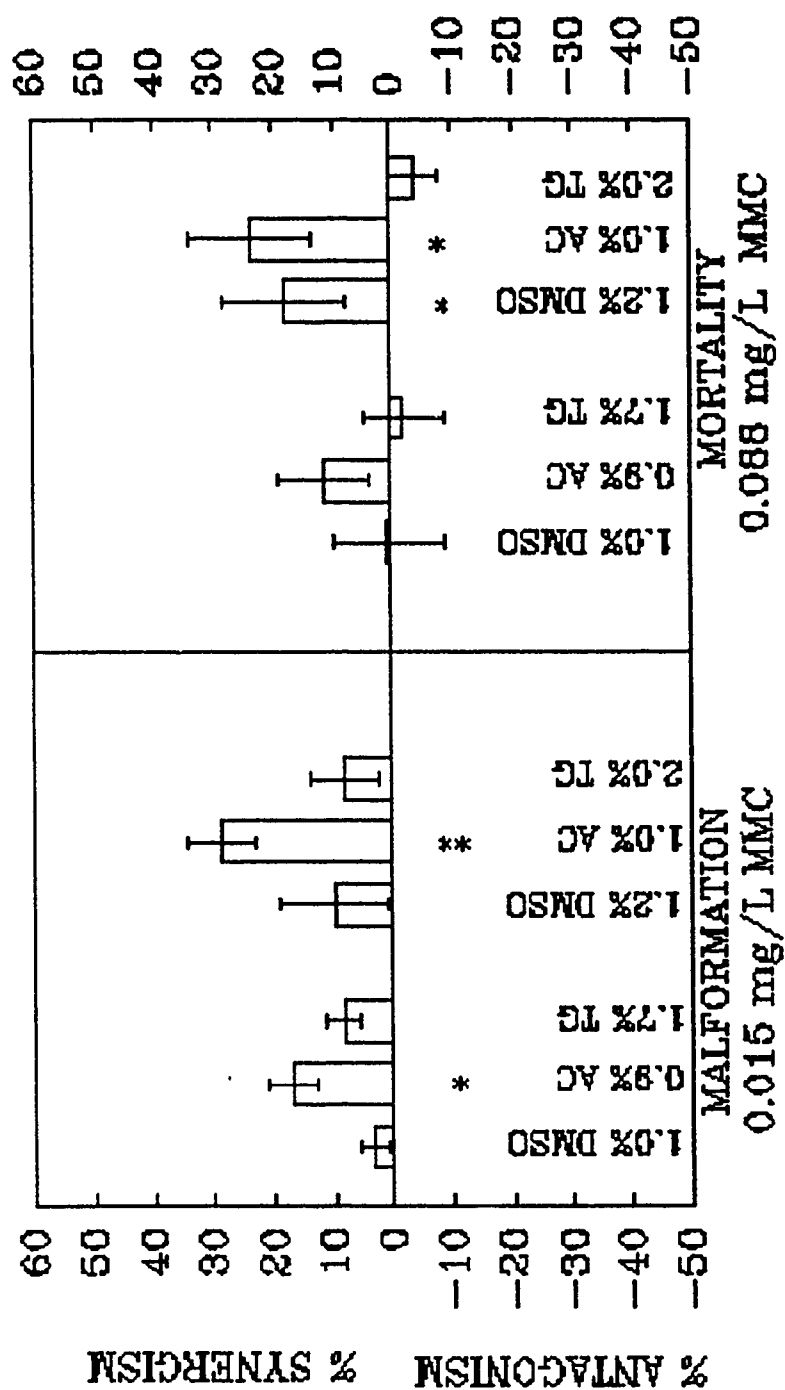


Figure 64. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for methylmercury chloride (MMC). Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments.

Figure 65

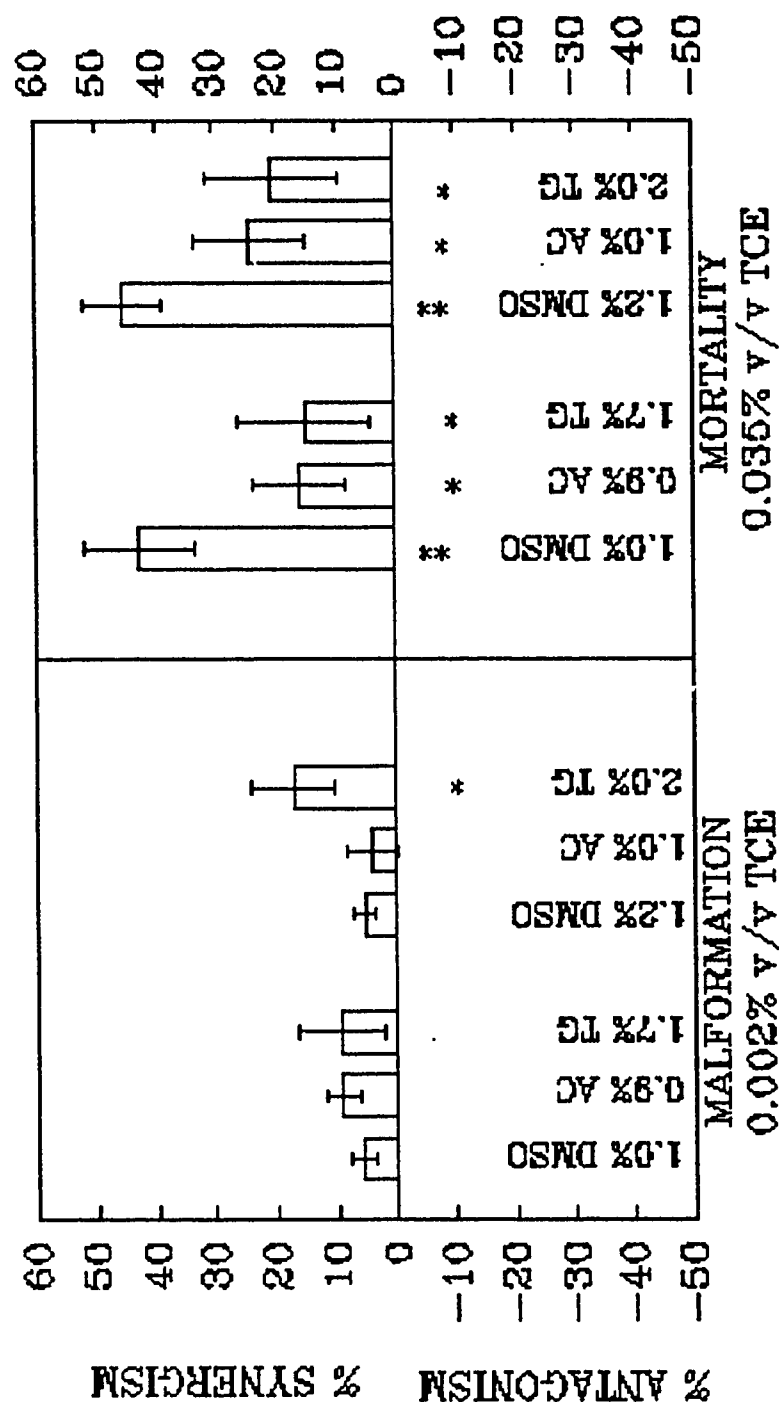


Figure 65. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for trichloroethylene (TCE). Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments.

Figure 66.

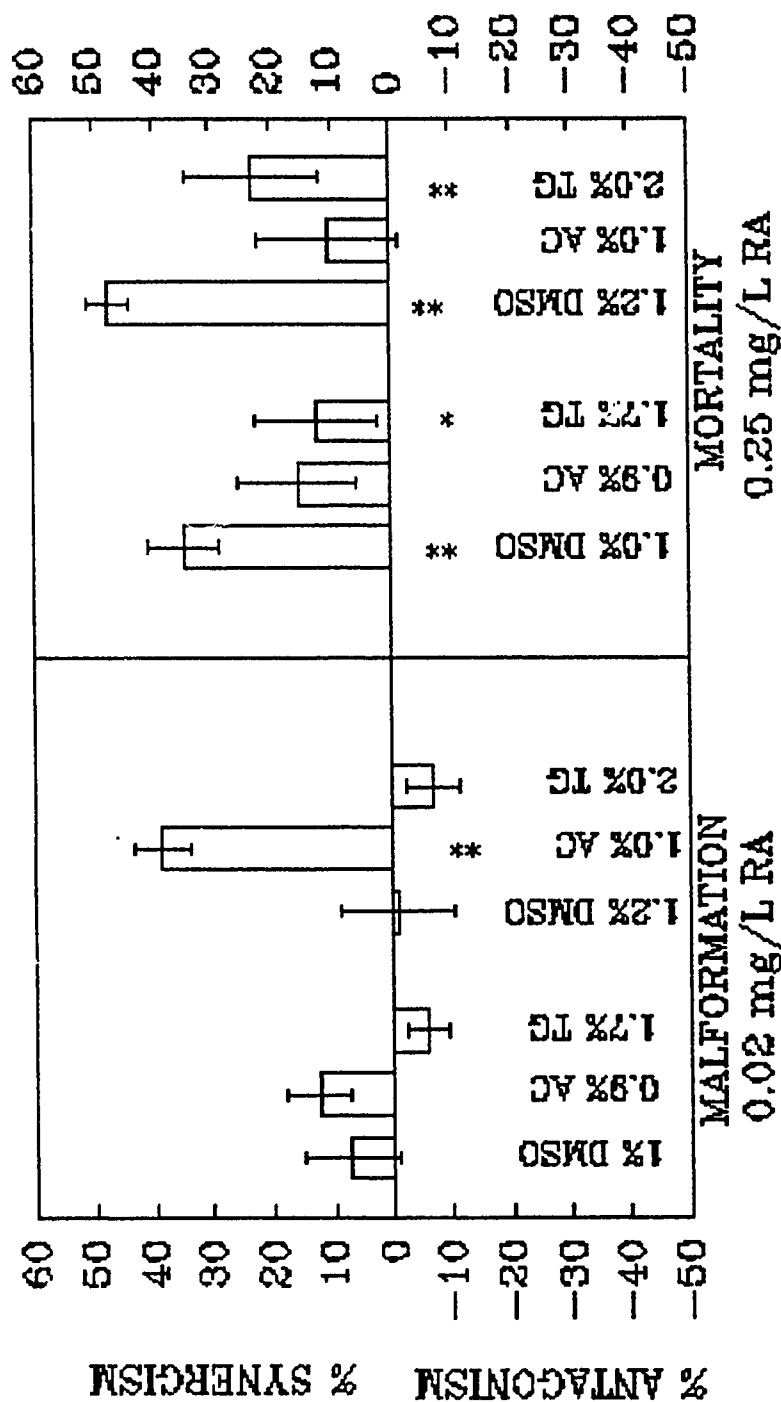


Figure 66. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for retinoic acid (RA). Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at the p=0.05 and ** = significantly different at p=0.001. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments and is why two close values are significantly different.

Figure 67

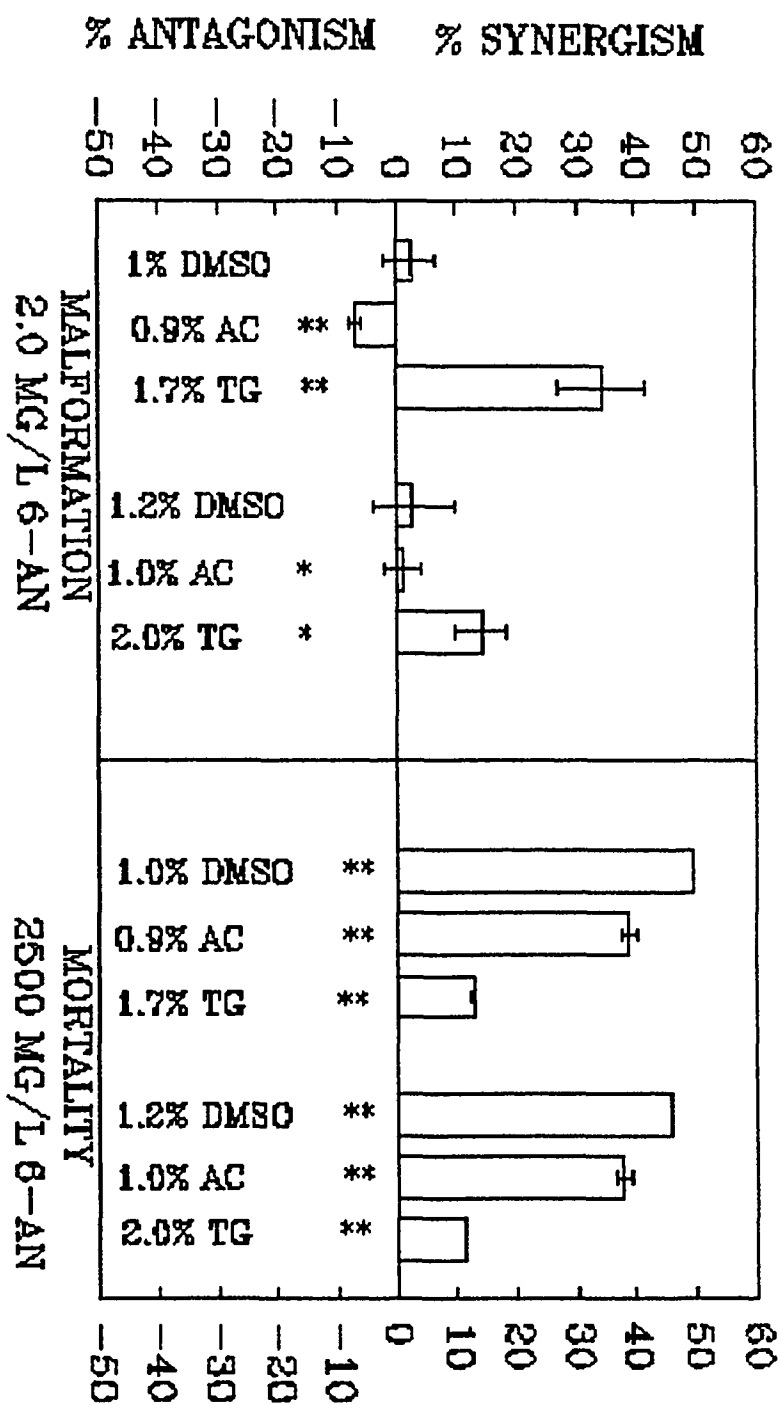


Figure 67. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for 6-aminonicotinamide (6-AN). Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at the $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments and is why two close values are significantly different.

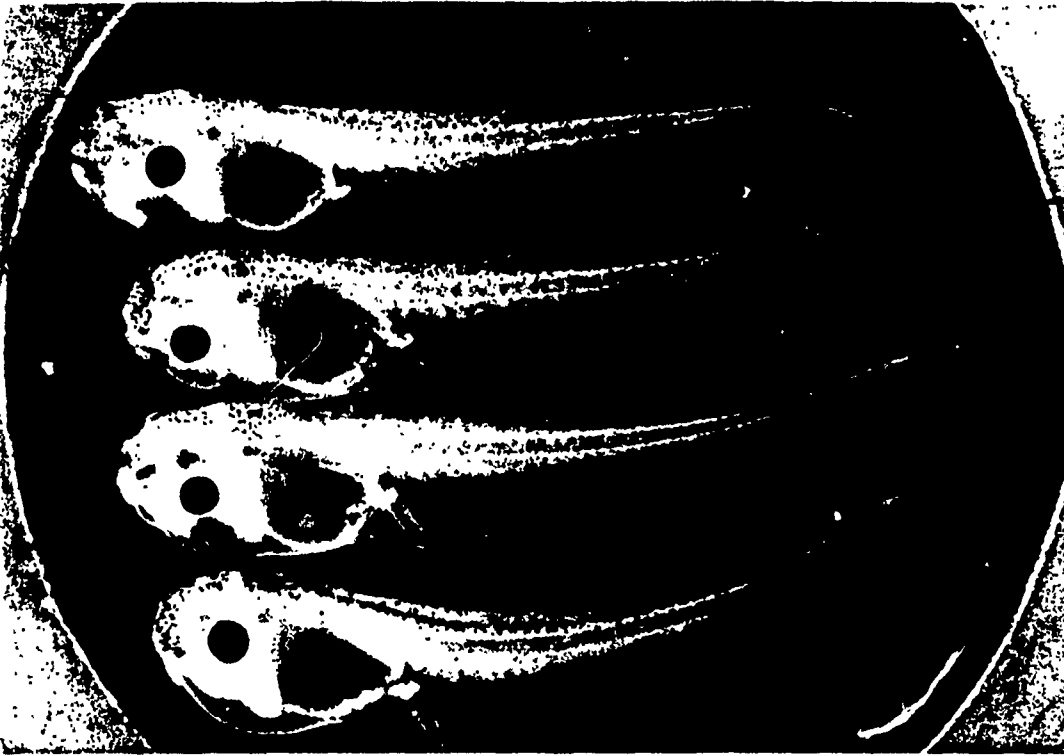


Plate 28. Side view, from top to bottom Control, RA 0.02 mg/L, DMSO 1.2% v/v, RA 0.02 mg/L & DMSO 1.2% (v/v). Note blunting of head of RA larva, and slight tail muscular kinking of DMSO larva. The combined embryo shows both blunting and tail muscular kinking.



Plate 29. Ventral View, from left to right Control, RA 0.02 mg/L, DMSO 1.2% v/v, RA 0.02 mg/L & DMSO 1.2% v/v. Both RA and DMSO induce loose gut coiling individually and together.

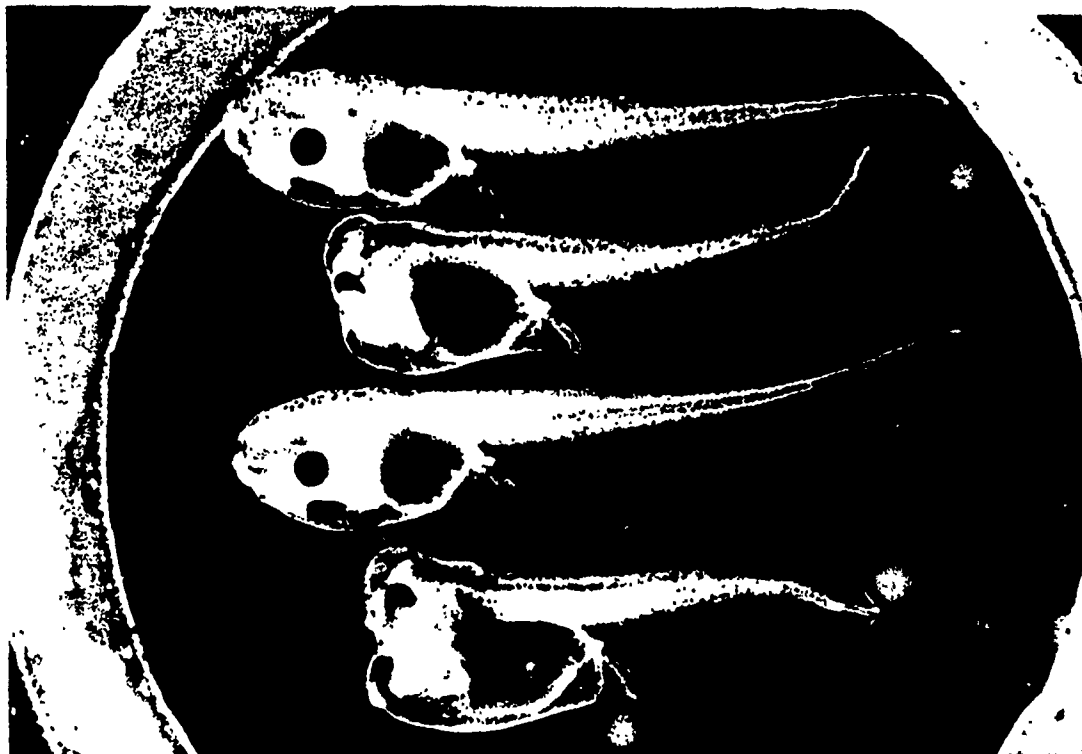


Plate 30. Side view, from top to bottom, Control, RA 0.25 mg/L , DMSO 1.0% v/v, RA 0.25 mg/L & DMSO 1.0% v/v. The serverity of the embryo is not changed due to the addition of DMSO 1.0%.



Plate 31. Ventral view, from right to left, Control, RA 0.025 mg/L, DMSO 1.0%, RA 0.25 mg/L & DMSO 1.0%.



Plate 32. Side view, from top to bottom, Control, RA 0.25 mg/L, DMSO 1.2% v/v, RA 0.25 mg/L & DMSO 1.2% v/v.



Plate 33. Ventral view, from right to left, Control, RA 0.25 mg/L, DMSO 1.2% v/v, RA 0.25 mg/L & DMSO 1.2% v/v.

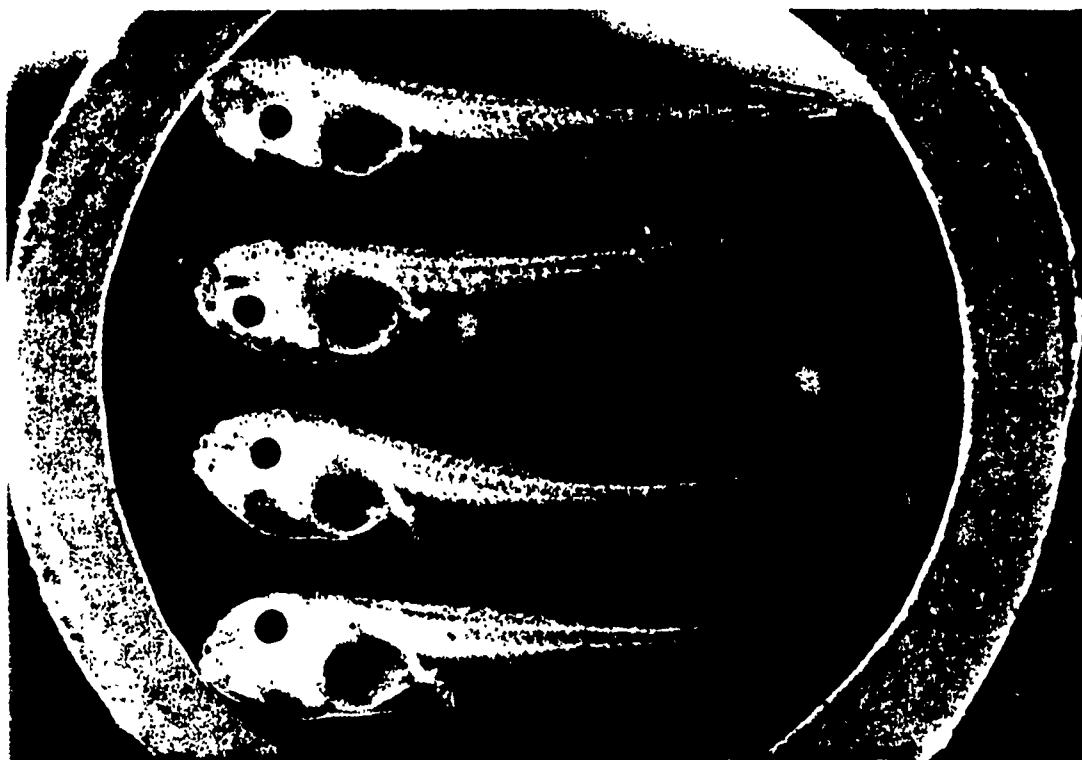


Plate 34. Side view, from top to bottom, Control, RA 0.02 mg/L, Acetone 0.9% v/v, RA 0.02 mg/L & Acetone 0.9% v/v.



Plate 35. Ventral view, from left to right, Control, RA 0.02 mg/L, Acetone 0.9% v/v, RA 0.02 mg/L & Acetone 0.9% v/v.



Plate 36. Side view, from top to bottom, Control, RA 0.02 mg/L, Acetone 1.0% v/v, RA 0.02 mg/L & Acetone 1.0% v/v.



Plate 37. Ventral view, from left to right, Control, RA 0.02 mg/L, Acetone 1.0% v/v, RA 0.02 mg/L & Acetone 1.0% v/v.

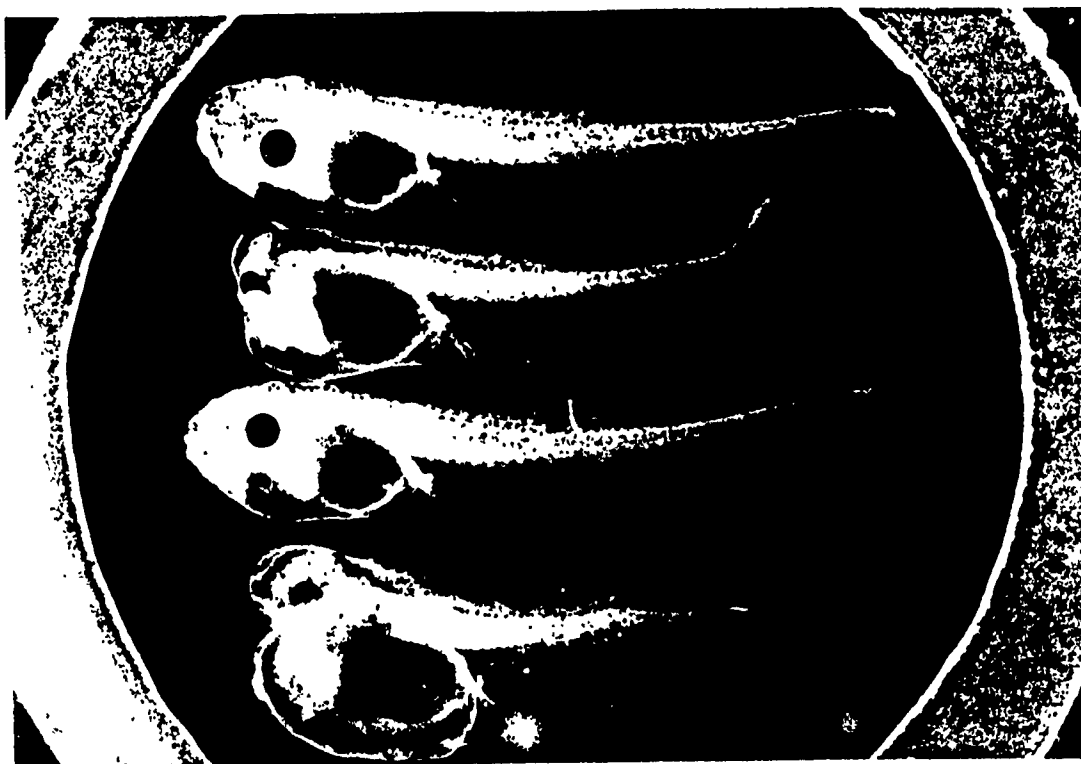


Plate 38. Side view, from top to bottom, Control, RA 0.25 mg/L, Acetone 0.9% v/v, RA 0.25 mg/L & Acetone 0.9% v/v.



Plate 39. Ventral view, from left to right, Control, RA 0.25 mg/L, Acetone 0.9% v/v, RA 0.25 mg/L & Acetone 0.9% v/v.

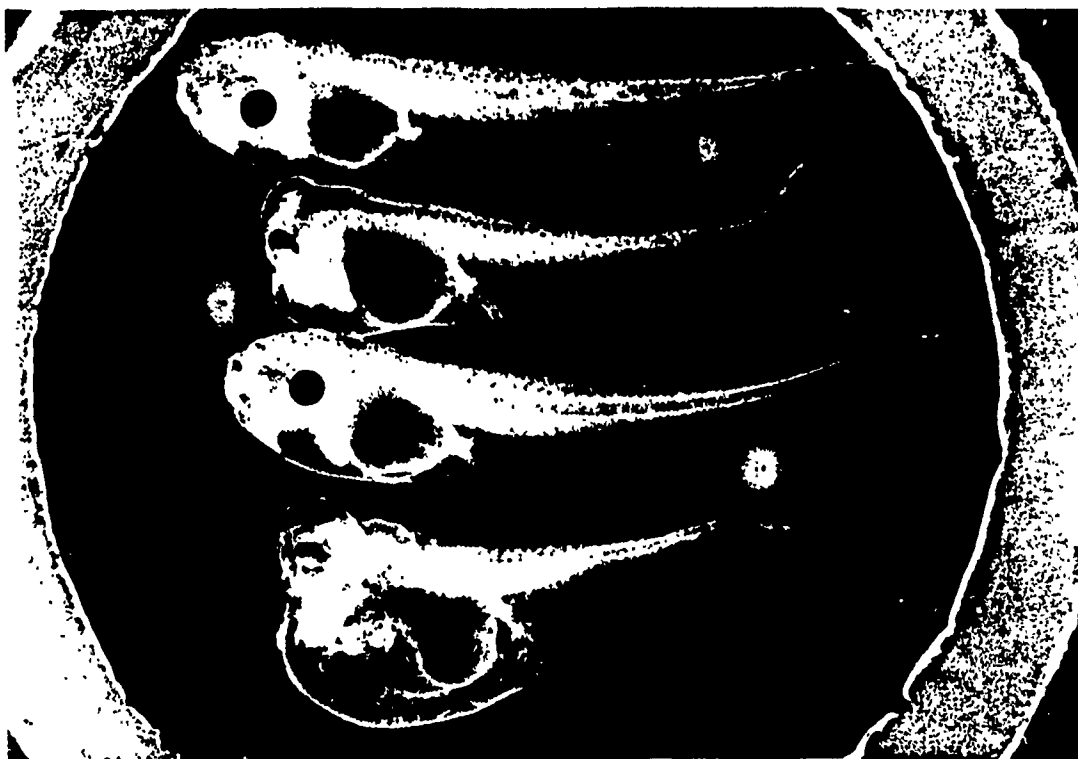


Plate 40. Side view, from top to bottom, Control, RA 0.25 mg/L, Acetone 1.0% v/v, RA 0.25 mg/L & Acetone 1.0% v/v.



Plate 41. Ventral view, from left to right, Control, RA 0.25 mg/L, Acetone 1.0% v/v, RA 0.25 mg/L & Acetone 1.0% v/v.

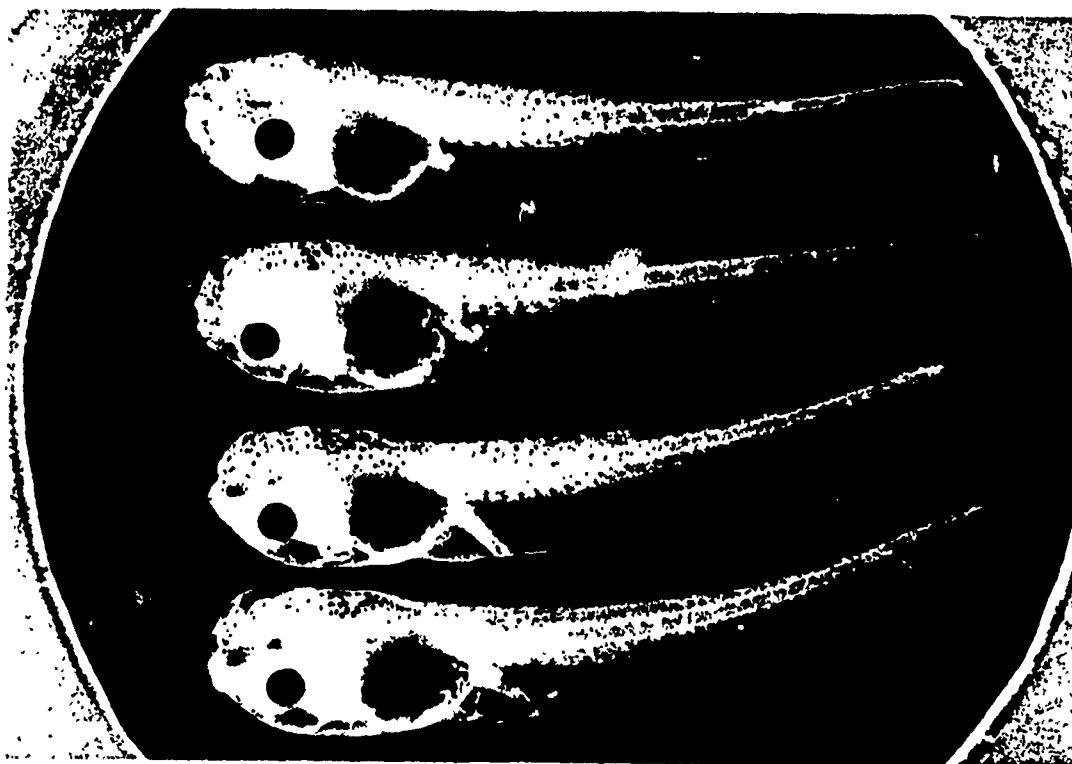


Plate 42. Side view, from top to bottom, Control, RA 0.02 mg/L, TG 2.0% v/v, RA 0.02 mg/L & TG 2.0% v/v.



Plate 43. Ventral view, from right to left, Control, RA 0.02 mg/L, TG 2.0% v/v, RA 0.02 mg/L & TG 2.0% v/v.

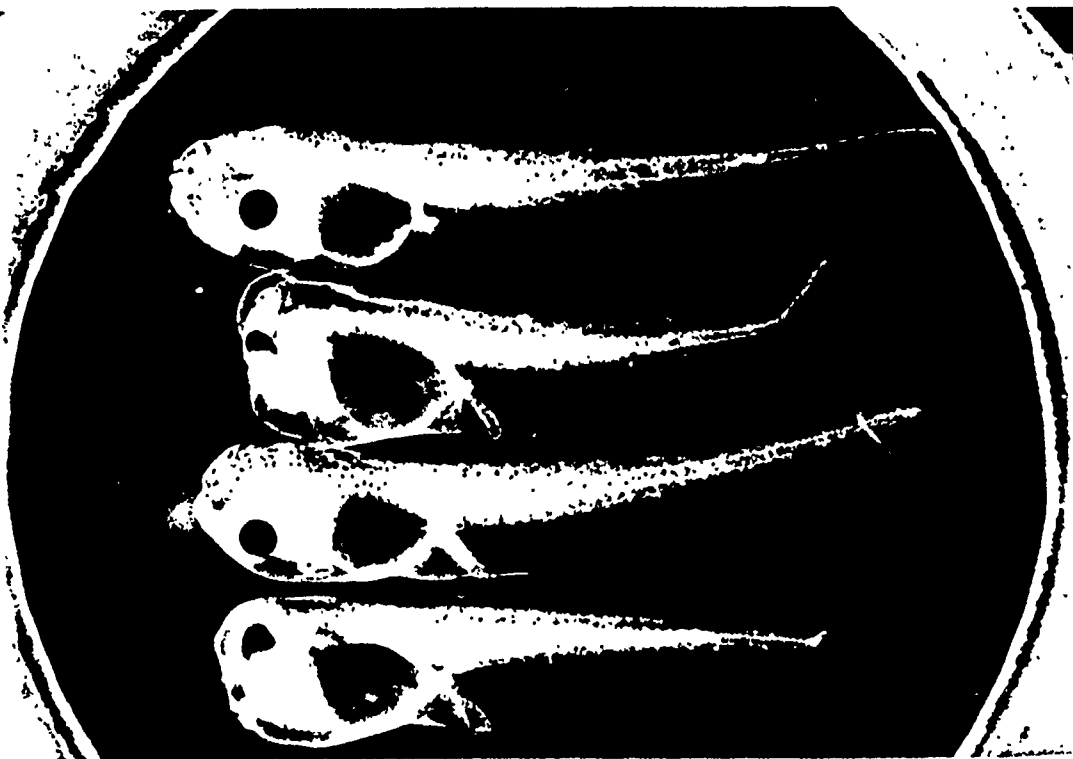


Plate 44. Side view, from top to bottom, Control, RA 0.25 mg/L, TG 1.7% v/v, RA 0.25 mg/L & TG 1.7% v/v.



Plate 45. Ventral view, from left to right, Control, RA 0.25 mg/L, TG 1.7% v/v, RA 0.25 mg/L & TG 1.7% v/v.



Plate 46. Side view, from top to bottom, Control, RA 0.25 mg/L, TG 2.0% v/v, RA 0.25 mg/L & TG 2.0% v/v.



Plate 47. Ventral view, from left to right, Control, RA 0.25 mg/L, TG 2.0% v/v, RA 0.25 mg/L & TG 2.0% v/v.

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Bantle, John A.

3. Metabolic Activation System

Acetaminophen: Acetaminophen (Tylenol) is listed as a variable negative that may be altered by metabolism (Table 3). Shepard (34) indicated that there was no evidence for human teratogenicity and most of the animal studies were negative. Potter et al. (44) presented evidence that cytochrome P-450 was involved in the conversion of Acetaminophen to an N-hydroxy derivative that causes hepatic necrosis in rats. This made the study of Acetaminophen interesting because it may be negative without an in vitro metabolic activation system (MAS) but positive with the rat liver MAS. Mammals may be able to ameliorate these negative effects because of the placental relationships.

Table 2 shows the effects of Acetaminophen on Xenopus growth and development. This compound has turned out to be very unusual in its mode of action. Although its solubility in aqueous solutions is excellent it nonetheless gave highly variable results. We have run this test 6 times with three different operators and continue to get unusual results. We only presented three of these experiments to conserve space. Dose-response curves undulate slightly (Figure 68) and the 96-hr LC50 can shift as is seen in the third definitive test with antibiotics. Some of the variability seen can be due to the presence of bacteria as the inclusion of penicillin and streptomycin improve embryo survival (definitive #3). However, interaction between antibiotic and Acetaminophen must be considered. We are continuing to explore this possibility in our lab. Acetaminophen is negative in FETAX for at least the majority of the tests conducted as the mean TI for definitives 1 and 2 is only 1.3. The mean MCIG is 0.11 and Figures 69 and 71 show that Acetaminophen does not affect growth until nearly 65% of the 96-hr LC50. Therefore, the best data available shows that Acetaminophen is a negative in FETAX when there is no metabolic activation system present. At this time we have not added the MAS to the experiment. If the mammalian literature is correct, the addition of rat liver microsomes may affect the 96-hr LC and EC50 (malformation) but this should not change the TI or the final conclusion that Acetaminophen is not a developmental toxicant. At this time we do not have any pictures of Acetaminophen treated embryos.

Bantle, John A.

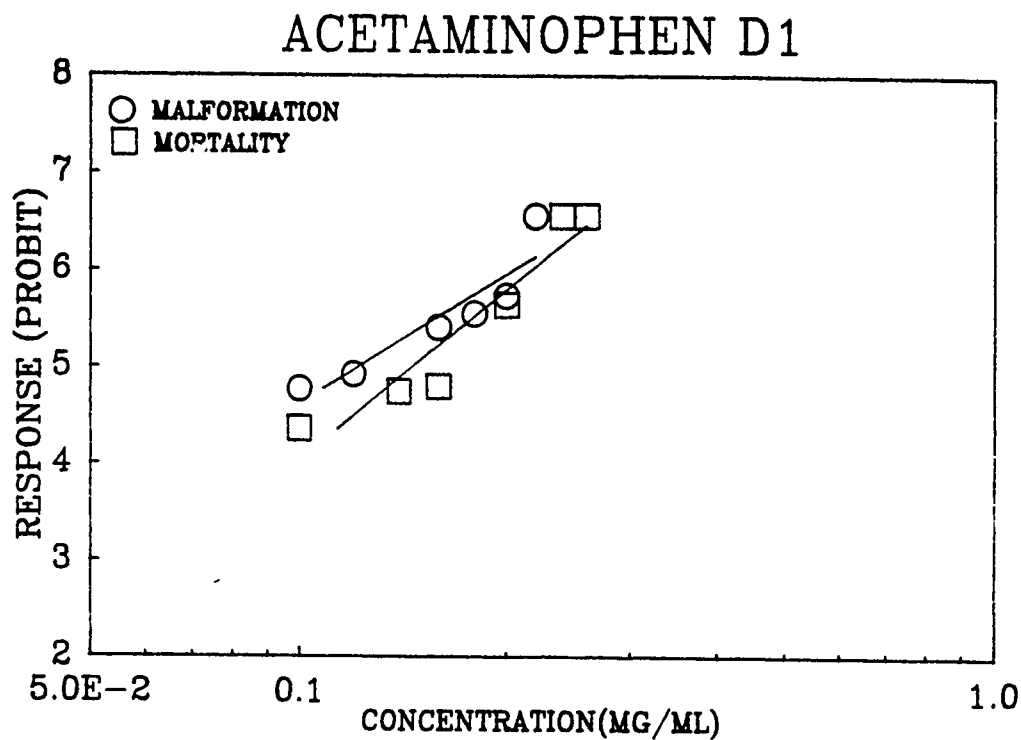


Figure 68. 96-h Mortality and Malformation Dose-Response Curves for Acetaminophen, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

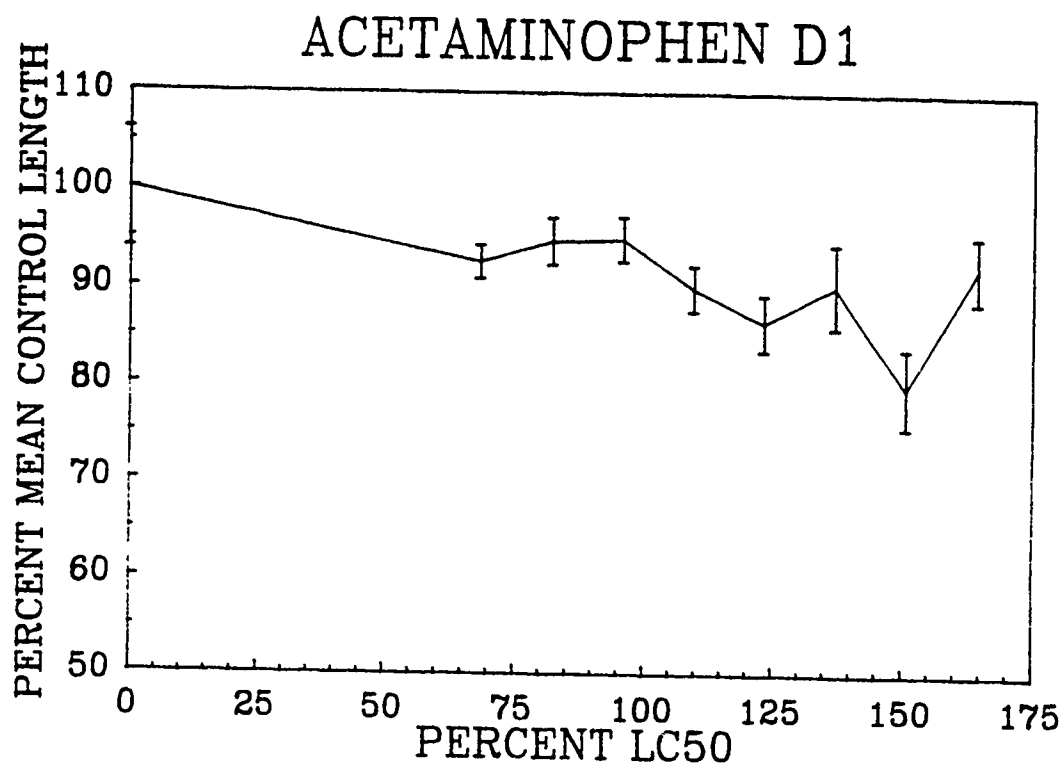


Figure 69. 96-h Growth Dose-Response Curve for Acetaminophen, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Bantle, John A.

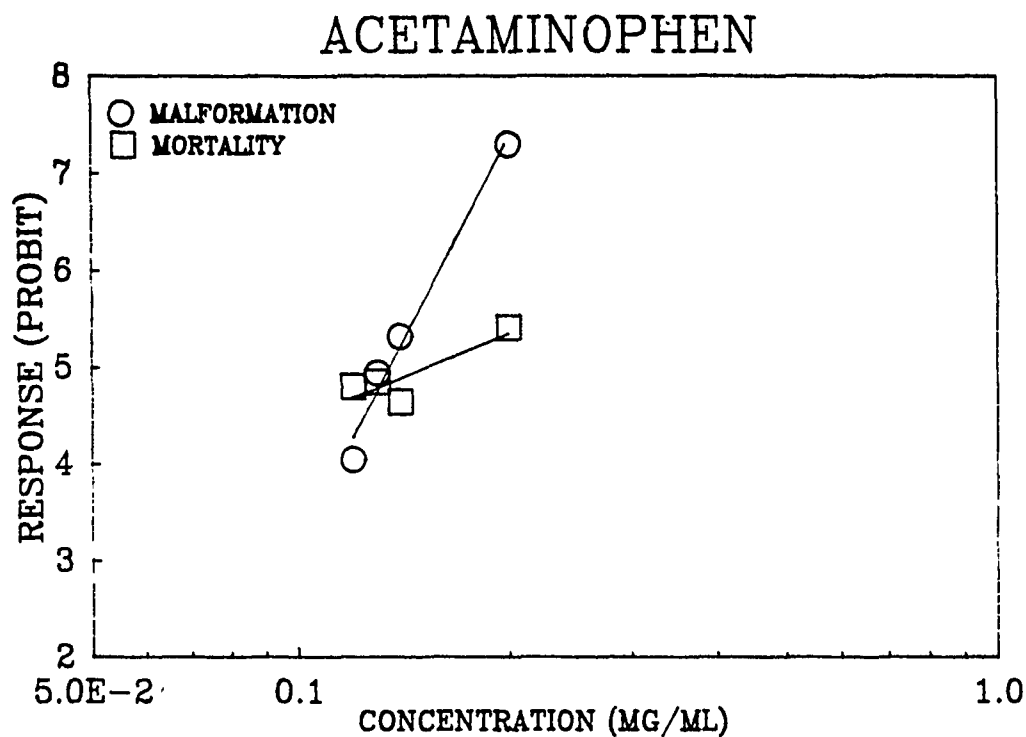


Figure 70. 96-h Mortality and Malformation Dose-Response Curves for Acetaminophen, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

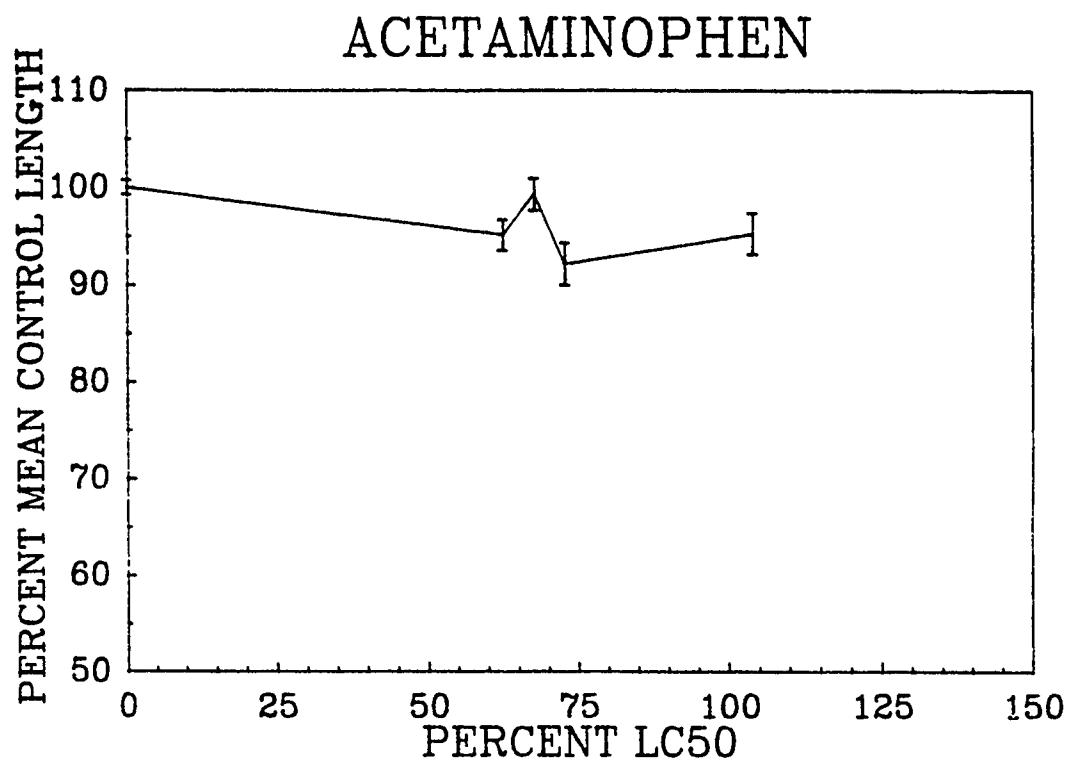


Figure 71. 96-h Growth Dose-Response Curve for Acetaminophen, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Bantle, John A.

Acetazolamide: We have only run a single range test on Acetazolamide to date (Table 3). Shepard (34) has 9 listings for Acetazolamide many of which indicate that Acetazolamide has significant developmental toxicity. Smith et al (22) provide evidence that Acetazolamide is a teratogen in rodents but not rabbits and monkeys. They also indicate that it is not metabolized. For this reason we listed it in Table 3 as a variable positive that was not subject to MAS. Sabourin and Faulk (14) reported a TI of <1.6 for Acetazolamide. However, Dumont obtained a TI of 3.46 for this compound. We have only performed a single range test without MAS to date and we used concentrations that were too low to observe an effect.

We have scheduled further work on this compound for the last quarter of the project.

Bantle, John A.

Benzo(a)pyrene: Benzo(a)pyrene (BP) is a common environmental contaminant which is often produced through combustion. Shepard (34) lists BP as a developmental toxicant with more resorptions (embryo lethality) occurring at higher concentrations than malformations. There is ample evidence that metabolic activation plays a crucial role in the conversion of BAP to an active metabolic that is mutagenic and able to bind to macromolecules such as DNA (45). We have listed BAP as a positive teratogen that requires metabolic activation for its activity (Table 3). The mean TI without MAS is listed in Table 3 as >1 but the value is really not able to be calculated because we could not get enough into solution to get an adequate 96-LC50. The mean 96-hr EC50(malformation) was 10 ug/ml without MAS. With the in vitro MAS, it was still not possible to get an 96-hr LC50 because of solubility problems but the 96 hr-EC50(malformation) was lowered to 1.17 ug/ml thereby increasing the spread between the theoretical mortality curve and the malformation dose-response curve. Figures 72 and 74 show two typical malformation dose-response curves for BP with and without MAS. It can be seen that bioactivation moves the curve towards lower concentrations. Figures 73 and 75 show only a modest increase in growth inhibition caused by the metabolic activation of BP.

In summation, metabolic activation of BP decreased the 96-hr EC50(malformation) by 5 to 6 fold. Embryo lethality was not affected up to the maximum soluble concentration. Thus, bioactivation significantly increased the potential hazard of BP. Unactivated BP induced primarily gut, mouth, and skeletal malformations (Plate 48). Bioactivation increased the severity of skeletal deformities and caused serious brain (microencephaly) and eye malformations at low BP concentrations. Some of the malformations induced by BP in mammalian test systems were similar to those observed with Xenopus. Shum et al. (46) found that B-naphthoflavone-enhanced BP metabolism in AKR inbred mice injected with BP between 50 and 300 mg/kg was associated with increased in utero toxicity and terata (club foot, cleft palate and lip, kinky tail, hemangioma, anophthalmia and scoliosis). Skeletal kinking in Xenopus may bear some relationship to skeletal limb defects in mammals. Similar terata were also observed in B6 (46) and C57BL/6 (47) strain mice after i.p. injection, but occurred more frequently than in the AKR strain. Greater incidence of anomalies found in the B6 strain has been attributed to genetic variability in the rate of BP biotransformation (i.e. C57BL/6N and B6 strain mice- a highly inducible P-450 isozyme (AAH) responsible for BP metabolism).

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BENZO(a)PYRENE I

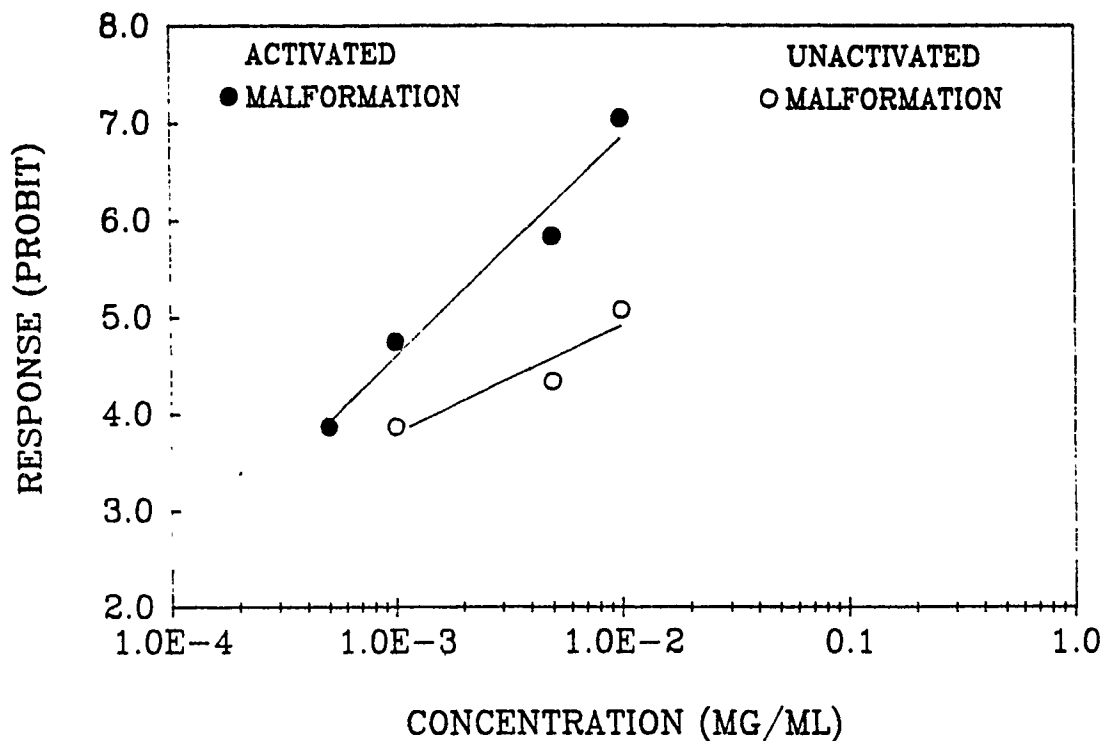


Figure 72 . 96-h Mortality and Malformation Dose-Response Curves for Benzo(a)pyrene Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

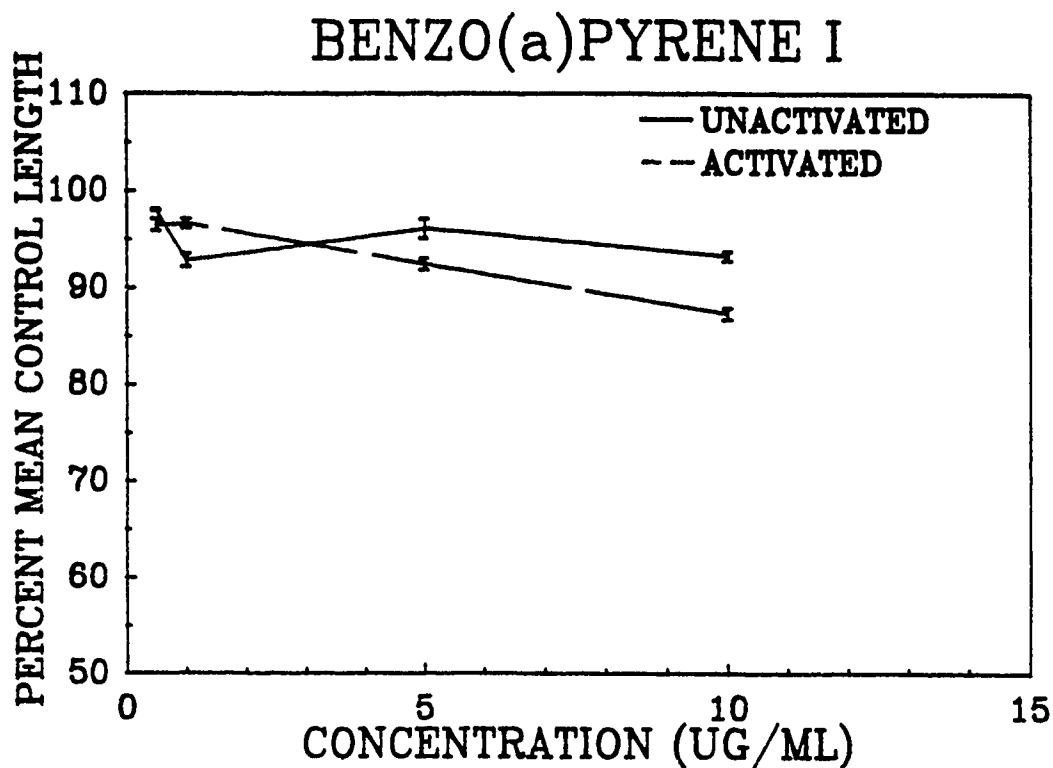


Figure 73. 96-h Growth Dose-Response Curve for Benzo(a)pyrene, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Bantle, John A.

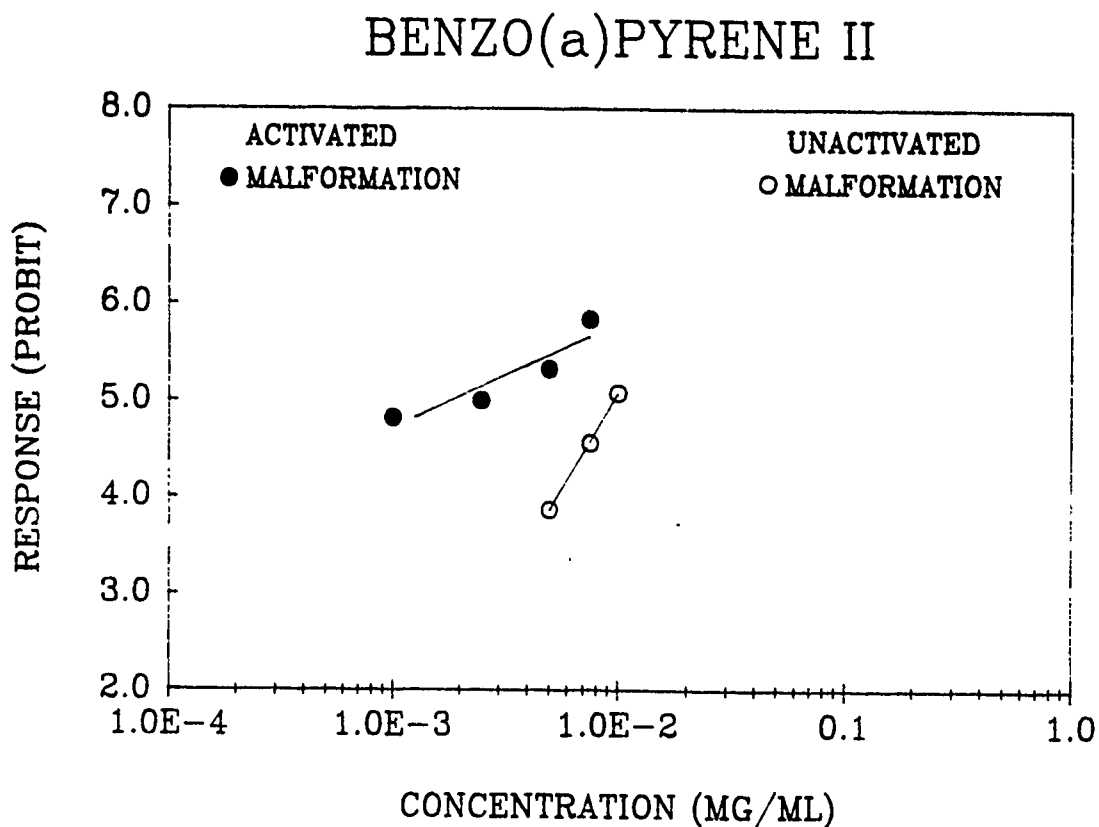


Figure 74. 96-h Mortality and Malformation Dose-Response Curves for Benzo(a)pyrene , Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

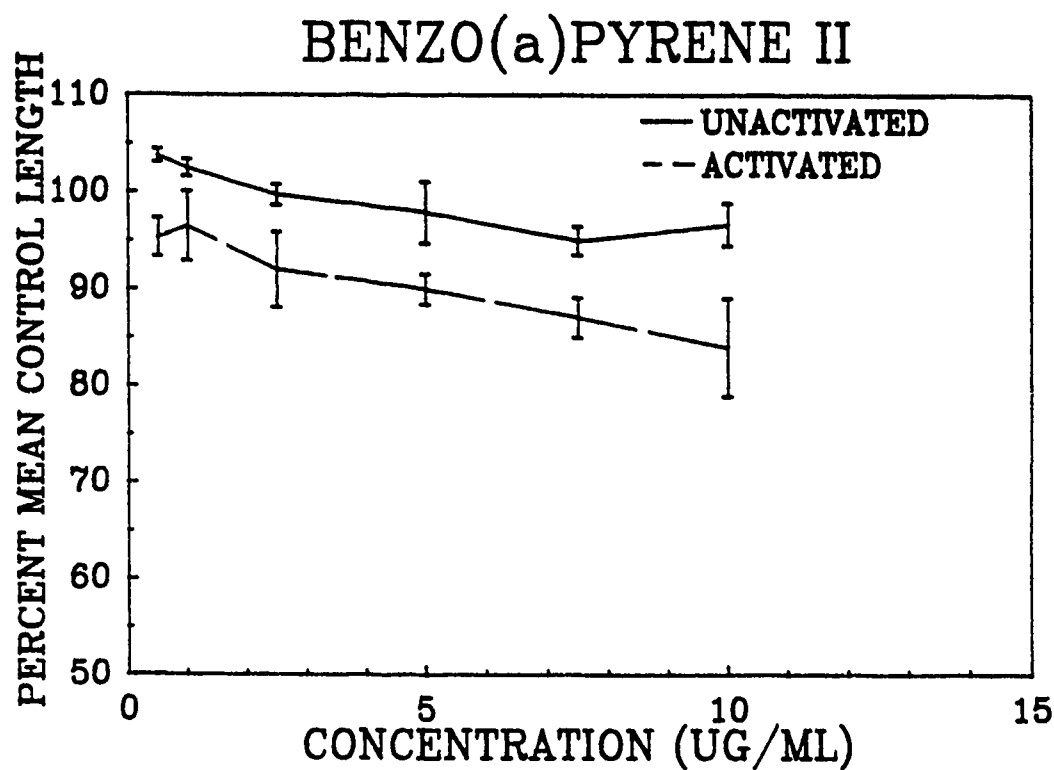


Figure 75. 96-h Growth Dose-Response Curve for Benzo(a)pyrene, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 48. Effects of Different Concentrations of Benzo(a)pyrene on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 10 ug/ml unactivated, 5 ug/ml activated.

Bantle, John A.

Dimethylnitrosoamine: Dimethylnitrosoamine (DMN) is a metabolically activated DNA alkylating agent that possesses considerable clastogenic (48), mutagenic (49) and teratogenic potential (50). DMN is probably metabolized to hydroxymethyl-methylnitrosamine. This developmentally toxic intermediate is very short lived. If the route of injection in mice is such that the dam can detoxify the substance before it gets to the embryo, then little teratogenicity occurs (50). When the route of injection is such that the DMN metabolite can be formed and promptly delivered to the embryo then it is a potent teratogen. This fact poses an unusual problem with FETAX. Since there is no maternal relationship and the metabolite should be formed just outside the Xenopus embryo by the rat liver microsomes, we would predict that unactivated DMN would be relatively nonteratogenic and that metabolized DMN would be about as teratogenic as Benzo(a)pyrene. This would be a perfectly logical finding but nonetheless a false positive result depending on the mammalian route of exposure. This points out the danger of using the mammalian database as a validation standard and the need to consider the metabolism of a toxicant before making a conclusion as to its hazard to human populations.

We have performed only a single test to date on DMN (Table 3). The results indicate that our original predictor is correct in the case of unactivated DMN. The TI is only 1.6 while the 96-hr LC50 and EC50(malformation) is 3.5 and 2.3 mg/ml respectively. Figure 76 shows the 96-hr dose-response curve for mortality and malformation. These curves are reasonably close together. It must be remembered that the 96 hr Xenopus embryo is attaining a degree of metabolic competence during the last stages of development. Thus, the two curves may be slightly wider as a result. Figure 77 shows the growth inhibition curve for DMN. Most of the growth inhibition observed occurs after 50% of the 96-hr LC50 concentration and after this point, the decline is quite sharp to 70% of control values. This growth inhibition curve is typical for a weak teratogen or growth inhibitor. By the next quarterly report we will have micrographs of the types of abnormalities and we will have evidence as to whether MAS causes a significant increase in developmental toxicity. If there is no significant increase then we will try an analog (Acetoxymethyl-Methylnitrosamine) which resembles the active intermediate. If FETAX responds to high doses of this intermediate then we must assume that DMN is quickly degraded on the outside by the rat liver microsomes and that the metabolite does not get into the embryo. If we do not get a response with this analog then we must assume that it either is not getting in or the embryo is not reacting to it.

Bantle, John A.

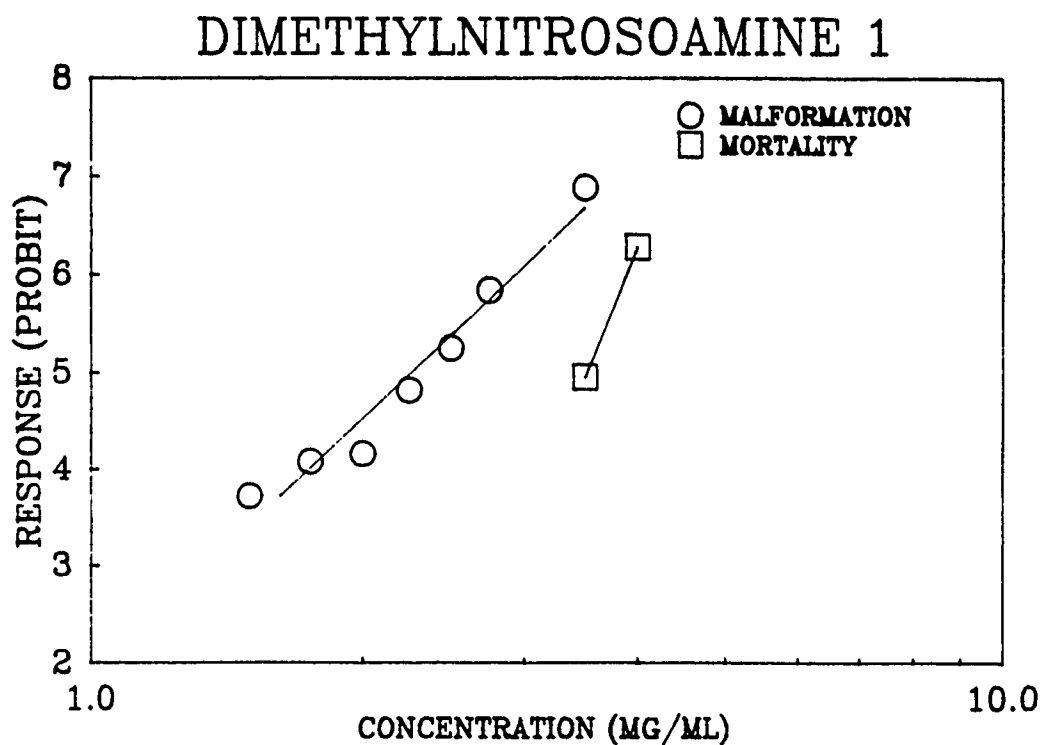


Figure 76. 96-h Mortality and Malformation Dose-Response Curves for Dimethylnitrosoamine, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

Bantle, John A.

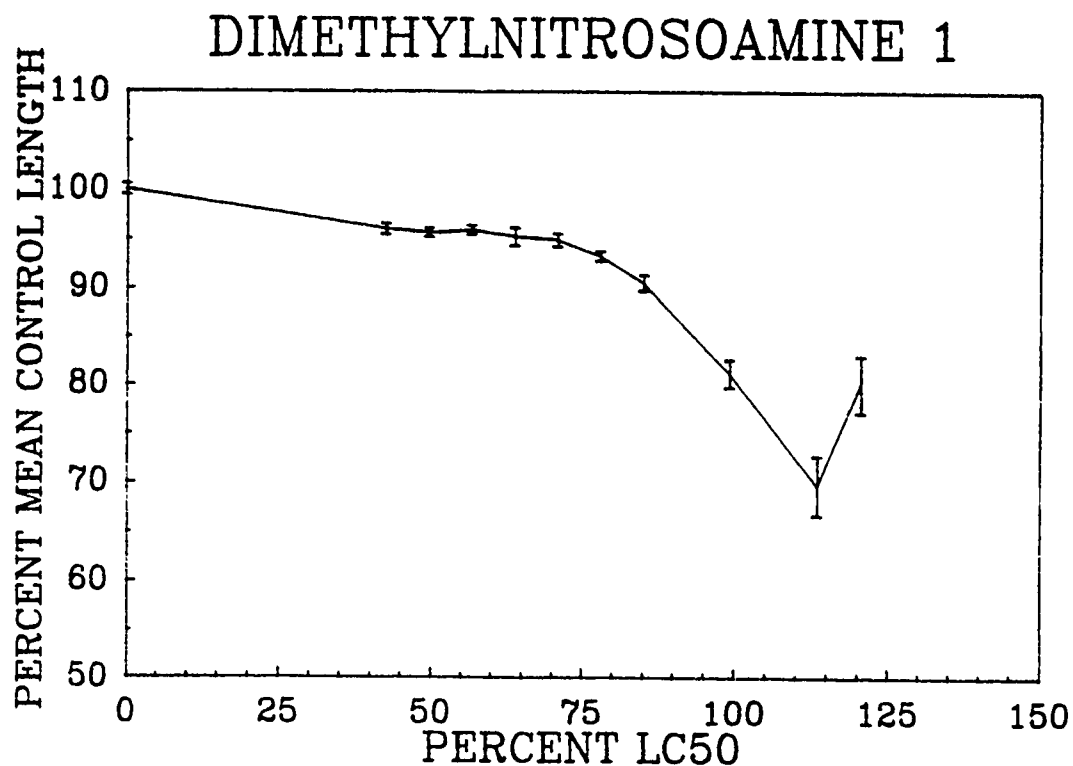


Figure 77. 96-h Growth Dose-Response Curve for Dimethylnitrosoamine, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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Sodium Salicylate: Sodium Salicylate is listed as a variable positive that should not undergo any metabolic activation (Table 3). There is little evidence presented in Shepard (34) that Sodium Salicylate causes terata in humans but it does cause abnormal development in many animal models. Smith et al. (22) list Aspirin, a related compound, as a strong animal positive teratogen but that there is no demonstrated teratogenicity in humans. There is also some variability in the literature on whether biotransformation plays a significant role in teratogenesis. Smith et al. (22) indicated that Aspirin is subjected to hydrolysis and detoxification by conjugation. Gabrielsson and Larsson (51) agree that it is the parent compound (in this case Salicylic Acid) that causes the teratogenic action and not its three major metabolites which do not cause harm when administered in pure form in rats. This data suggests that if biotransformation and conjugation is functional, the embryotoxicity, teratogenicity and growth inhibition of Sodium Salicylate should decrease. However, Juchau (52) has used Sodium Salicylate in the in vitro rat embryo culture system. This is the closest mammalian test system to FETAX plus rat liver microsomes because rat embryos can be cultured with and without S9 supernatant (A source of cytochrome P-450 enzymes). He reports that the addition of S9 to the culture system made little difference in the generation of teratogenicity in vitro. Because of this report we listed Sodium Salicylate as an expected negative for MAS in Table 3.

We found that the mean TI for Sodium Salicylate without MAS was 1.64 and the mean 96-hr LC50 and EC50(malformation) were 2.32 and 1.47 ug/ml respectively (Table 3). With the inclusion of a MAS to the basic FETAX protocol the mean TI dropped slightly to 1.55 and the mean 96-hr LC50 and EC50(malformation) were 2.25 and 1.45 ug/ml respectively. After an examination of the confidence limits for these dose-response curves (see: Data Summary sheets), we concluded as did Juchau (52) that the inclusion of a metabolic activation system had little effect on results. We were disappointed in seeing that the teratogenicity of Sodium Salicylate was very weak as the unactivated TI was only 1.64. However, Sabourin and Faulk (14) listed Aspirin as <1.6 and Dumont obtained a TI value of 1.43 for Sodium Salicylate so we think that our value is correct. Figures 78 and 80 show the dose-response curves for the two tests on this compound. The tests for activated Sodium Salicylate were more repetitive than for unactivated. Figures 79 and 81 show the effect of Sodium Salicylate on embryonic growth. Once again the inclusion of MAS makes little difference in Figure 79 and a greater difference in the higher concentration regions of Figure 81. The inability to cause significant growth inhibition at concentrations below 50% of the 96-hr LC50 is more suggest of a weak or nonteratogen than a strong teratogen. Plate 49 shows that malformations caused by Sodium Salicylate can be quite severe. Whether exposed to Sodium Salicylate with MAS or without, similar types of malformations are observed. Blistering, edema and severe malformations to all major body organs are evident. However, tail kinking is minimal. We have thus far concluded that Sodium Salicylate is not detoxified well in our system and obviously it is not eliminate as it is in mammals. We do a better job of repeating the cultured rat embryo test system than we do the in vivo model.

In summary, our results so far using rat liver microsomes are encouraging. It should be possible to reduce the number of false positives and negatives in FETAX by employing Aroclor 1254 induced rat liver microsomes as an in vitro metabolic activation system. Given that these microsomes are only a subset of all the mammalian detoxification systems, there will be some compounds that still do not test as expected.

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SALICYLIC ACID I

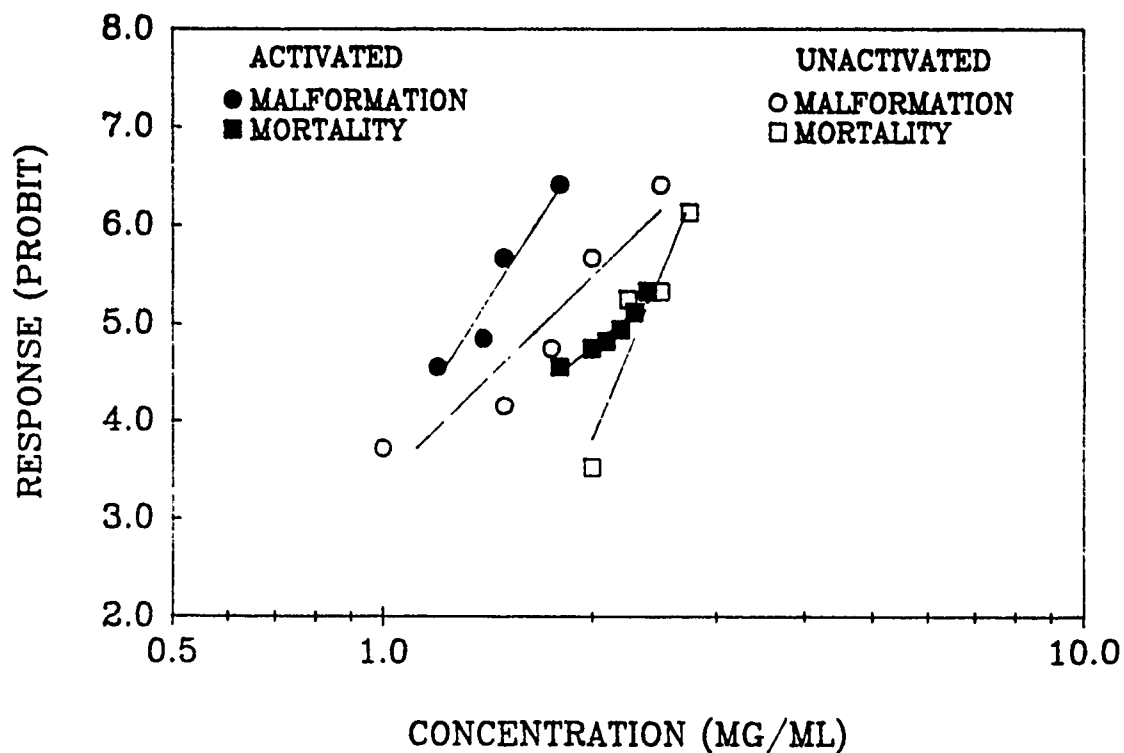


Figure 7a. 96-h Mortality and Malformation Dose-Response Curves for Salicylic Acid, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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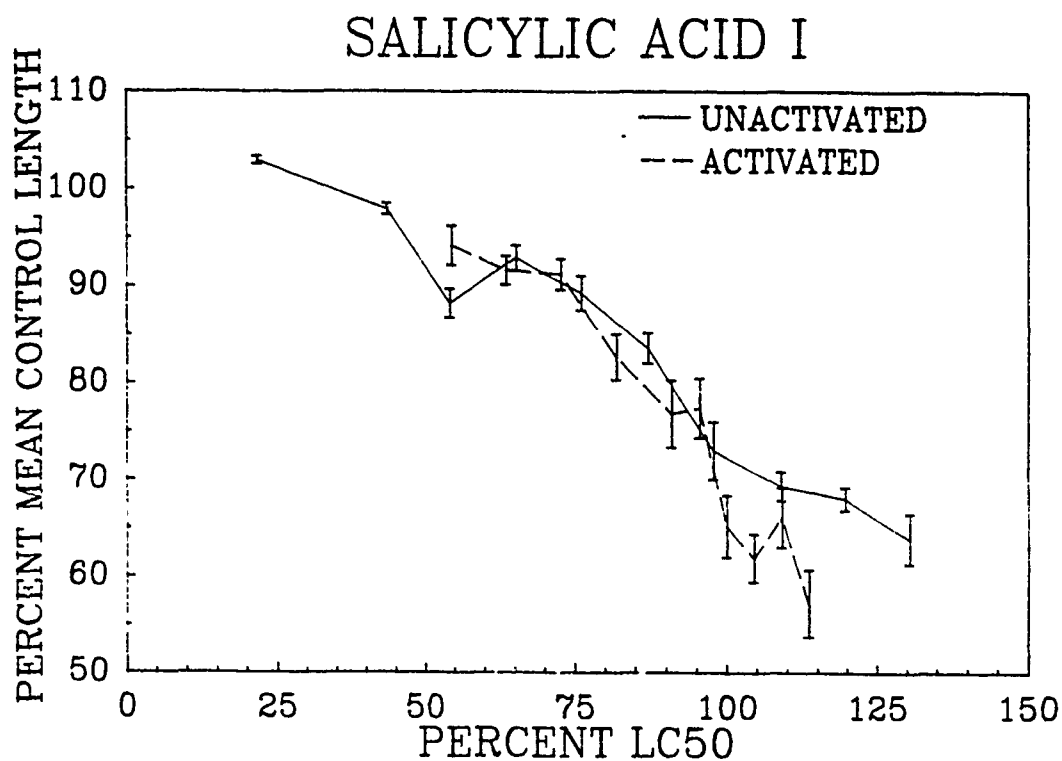


Figure 79 . 96-h Growth Dose-Response Curve for Salicylic Acid, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

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SALICYLIC ACID II

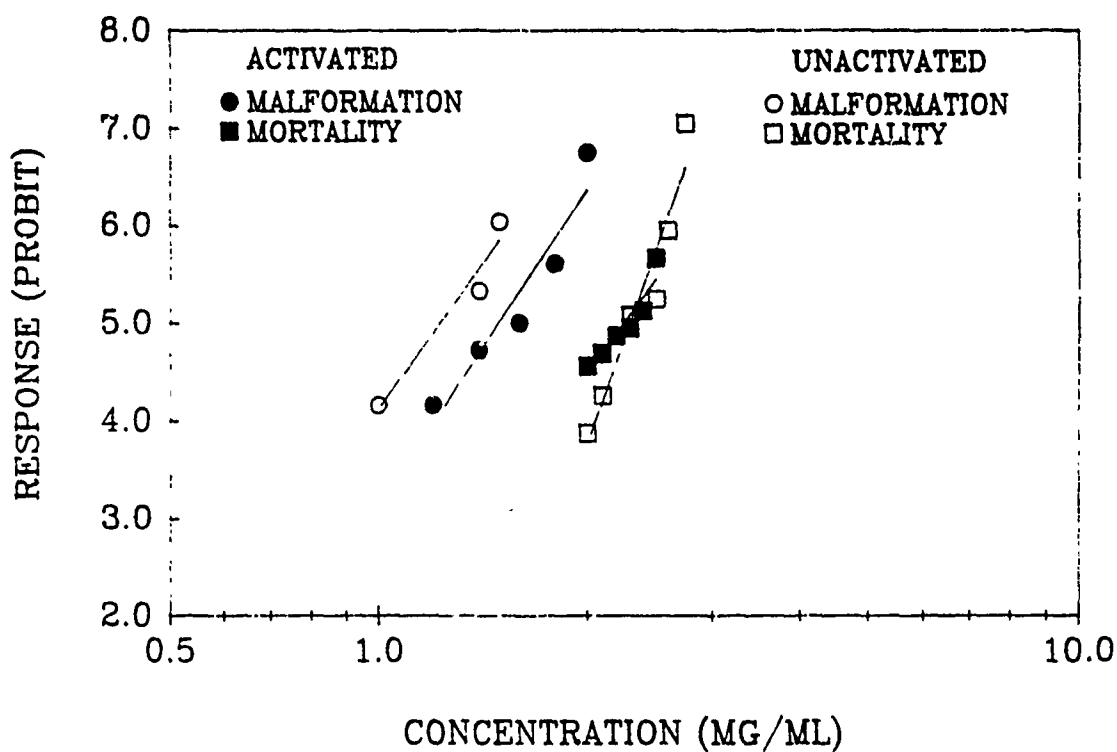


Figure 80. 96-h Mortality and Malformation Dose-Response Curves for Salicylic Acid, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well (See: Appendix). Each point represents 50 embryos for each concentration.

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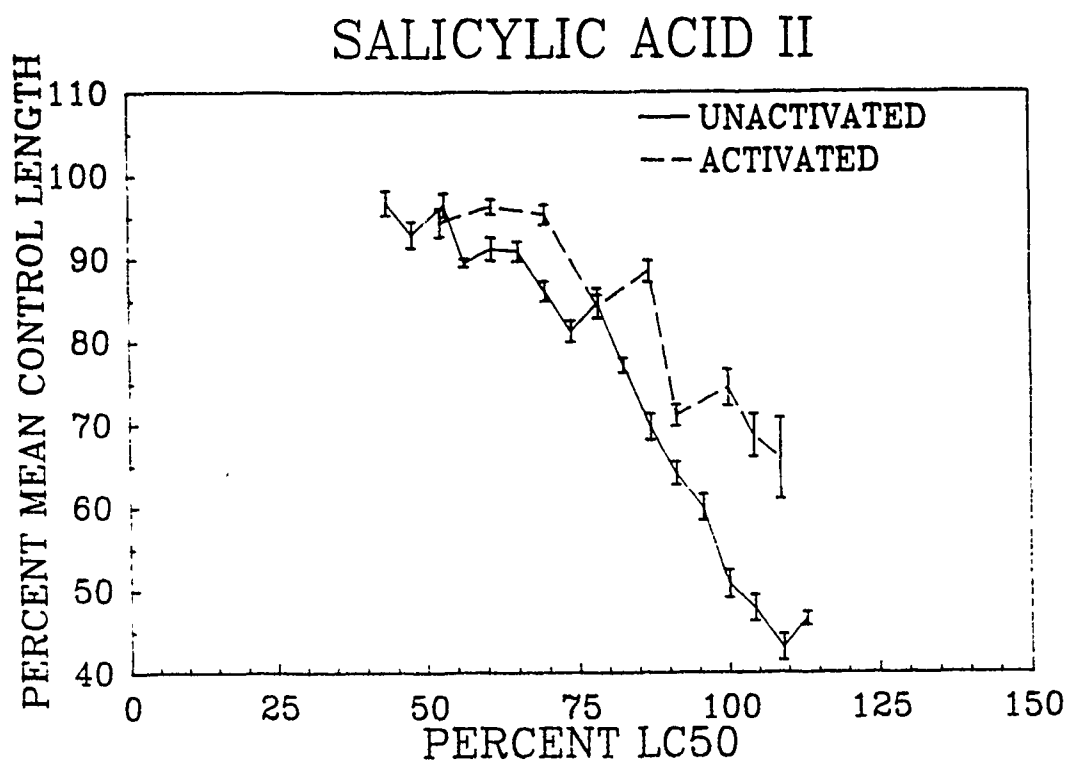


Figure 81. 96-h Growth Dose-Response Curve for Salicylic Acid, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.



Plate 49. Effects of Different Concentrations of Sodium Salicylate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2 ug/ml unactivated, 2 ug/ml activated.

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COMPARATIVE EFFECTS OF SODIUM ACETATE, CAFFEINE, AND
5-FLUOROURACIL ON THE DEVELOPMENT OF FROG (XENOPUS LAEVIS) AND
FATHEAD MINNOW (PIMEPHALES PROMELAS) EMBRYOS

The developmental toxicities of sodium acetate (SA), caffeine (CAF), and 5-fluorouracil (FLU) were determined by examining malformation, mortality, and growth of exposed frog (Xenopus laevis) and fathead minnow (Pimephales promelas) embryos. The tests were performed using a modified protocol of the Frog Embryo Teratogenesis Assay--Xenopus (FETAX). This protocol allowed for the same temperature and exposure time for both species (120 hrs or 5 days) and for similar embryological events to occur during exposure.

The fathead minnow (Pimephales promelas) has been established in many laboratories as an assay fish for determining the toxicity of complex environmental mixtures and pure compounds (Brungs, 1969; Devlin et al., 1985; Holcombe et al., 1982; McKim, 1977; Pickering and Gast, 1972). When combined, the use of fish and amphibian embryos together as test organisms are useful for evaluating the developmental toxicity of chemicals and environmental mixtures (Birge et al., 1983; Birge et al., 1985; Dawson et al., 1988).

Because of the number of tests performed with aquatic organisms, an interspecies comparison study is necessary to further investigate the effects that toxicants have on different species. It is impossible to compare sensitivities when the test organisms have been exposed during different embryological stages. Differing exposure periods (time) can strongly influence bioassay results as the assumption that internal toxicant levels are equal to the toxicant-water concentration is only true when steady-state is reached (McCarty, et al. 1985; McCarty, 1986; Veith et al. 1979).

In this investigation a modified FETAX protocol was followed in order to determine the relative developmental toxicities of sodium acetate, caffeine, and 5-fluorouracil to frog (Xenopus laevis) and fathead minnow (Pimephales promelas) embryos. Previous studies with zinc employing a 4 day exposure for Xenopus and 6 day exposure for Pimephales found that Pimephales were more sensitive than Xenopus (Dawson, et al. 1985). Birge et al. (1979;1983) have attempted to make amphibian and fish species comparisons on development and exposure but have used differing exposure periods (time to hatching plus four days).

The purpose of this study and the choice of the two species involved is to derive comparable data and to determine if species differences can be attributed to relative uptake of toxicant. Endpoints measured include growth, malformation, and mortality. A Teratogenic Index [TI=120-hr LC50/120-hr EC50 (malformation)] was calculated for each test. A species can be more sensitive by showing affects at lower concentrations, or it can also show a greater TI and thus be more sensitive to developmental toxicants.

MATERIALS AND METHODS

Chemicals

Pure chemicals tested included sodium acetate (SA) a nonteratogen, caffeine (CAF) a moderate teratogen and 5-fluorouracil (FLU) a strong teratogen. These were chosen based on their developmental toxicity, availability, and known mammalian data (Smith et al., 1983). Chemicals were obtained for initial testing from Sigma Chemical Co.

Test Organisms

Animal culture and breeding procedures for Xenopus were as described previously (Dawson and Bantle, 1987). Frog eggs were sorted for normally developing fine-cell blastulae to early gastrula stage eggs (Nieuwkoop, 1975). Fathead minnow eggs were obtained the day of the test from the Water Quality Research Laboratory, Oklahoma State University, and from Stover and Associates, Biometrics Laboratory, Stillwater, Oklahoma. Fish eggs were separated and chosen for fine-cell blastula to early gastrula stages (Devlin, 1982).

Initial Concentration-Response Studies

Dilutions of the test material were made with modified FETAX solution (MFS) which has been shown to allow normal development of both frog and fathead minnow embryos (Dawson, 1988). MFS is a reconstituted water medium containing 400 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 14 mg CaCl₂, 60 mg CaSO₄·2H₂O, and 75 mg MgSO₄ per liter of deionized distilled water.

At least one range test and two definitive tests were conducted for each compound. For the range tests at each dilution 20 frog embryos and 10 or 15 fish embryos were exposed. For the definitive tests at each dilution 20 frog embryos and 15 or 20 fish embryos were exposed. Tests were composed of four dishes of controls and two dishes of each concentration with 8 ml total solution.

Static renewal tests were conducted for 5 days (120 hrs) at 24°C ± 2 for both species. Test organisms were incubated with a photoperiod of 16 hr light and 8 hr darkness. A slight variability in temperature was due to lighting which was necessary to allow for maximum fish hatching. During the tests pH was measured daily and dead organisms were counted and removed.

At termination of the test surviving organisms were anesthetized with 3-aminobenzoic acid ethyl ester (methanesulfonate salt) and fixed with 3.0% (w/v) formalin. The number and type of gross terata occurring were determined with a dissecting microscope. Head-to-tail length of surviving Xenopus and hatched fish was collected using an IBM-compatible computer equipped with digitizing software (Jandel Scientific, Corte Madera, CA).

The concentration inducing malformation in 50% of surviving embryos (120hr-EC50) and the 120hr-LC50 were determined using

Litchfield-Wilcoxin probit analysis (Tallarida and Murray, 1980). In addition, the 96hr-LC50 was determined for each test to see if significant death occurred between day 4 and day 5. The t-test for grouped observations was used to determine the Minimum Concentration to Inhibit Growth (MCIG) in each test ($p = 0.05$).

In this investigation the frog control mortality and malformation rates were 50 of 560 (8.9%) and 16 of the 510 survivors (3.1%), respectively. For fish the control mortality and malformation rates were 18 of 460 (3.9%) and 14 of the 442 survivors (3.2%), respectively. Acceptable rates of control mortality and malformation in FETAX are generally $< 10\%$.

RESULTS

1. Sodium acetate

Sodium acetate did not demonstrate teratogenicity to either Xenopus or Pimephales. Xenopus mortality and malformation were affected at concentrations lower than for fish. Similar malformations were observed for both. (Plates 50-52)

The 120-hr EC50 (malformation) for Xenopus embryos exposed to SA was 3.3 mg/ml. Variable results were seen between 3.0-5.0 mg/ml because of tank effects. The 120-hr LC50 was 4.3 mg/ml. The resulting TI was 1.3 and the MCIG was 3.5 mg/ml. The most common malformations induced by SA in frog embryos was failure of the gut to coil along with optic and facial malformations, and edema. These malformations were evident at concentrations > 2.0 mg/ml. At concentrations > 3.5 mg/ml spinal kinking and stunting was common. At concentrations > 4.5 mg/ml, severe kinking, optic and facial malformations and edema occurred.

The 120-hr EC50 (malformation) for fathead minnow embryos exposed to SA ranged from 13.3-13.5 mg/ml. The 120-hr LC50 ranged from 9.0 to 9.2 mg/ml. The resulting TI was 1.5. The MCIG ranged from 7.0 to 8.0 mg/ml. The most common malformation induced by SA in fish was spinal kinking and stunting. At concentrations > 7 mg/ml spinal kinking and stunting was common along with heart edema and facial malformations. At concentrations > 13 mg/ml severe kinking, optic and facial malformations and edema occurred.

2. Caffeine

Caffeine was only slightly teratogenic to Xenopus but was strongly teratogenic to fish. Pimephales were malformed at concentrations lower than that required to effect Xenopus. Fish also survived to concentrations higher than Xenopus could tolerate--thus making the TI for fish greater. Both organisms demonstrated similar trends in malformation, though fish exhibited more severe anomalies as in complete curling of the tail. (Plates 53-55)

The 120-hr EC50 (malformation) for Xenopus embryos exposed to CAF ranged from 0.12 to 0.13 mg/ml. The 120-hr LC50 ranged from 0.18 to 0.22 mg/ml. The resulting TI was 1.5-1.7 and the MCIG ranged from 0.05 to 0.10 mg/ml. The most common malformations induced by CAF in frog embryos was spinal kinking

at concentrations > 0.03 mg/ml. At concentrations > 0.14 mg/ml, facial malformations, and improper gut coiling were common. At concentrations > 0.16 mg/ml moderately severe spinal kinking, stunting, and edema were observed.

The 120-hr EC50 (malformation) for fathead minnow embryos exposed to CAF ranged from 0.04 to 0.10 mg/ml. The 120-hr LC50 ranged from 0.51 to 0.76 mg/ml. The resulting TI was 7.6-12.8 and the MCIG was < 0.02 mg/ml. The most common malformations induced by CAF in fish were spinal kinking and stunting at concentrations > 0.02 mg/ml along with occasional facial and eye malformations. At concentrations > 0.10 mg/ml moderate spinal kinking, heart defects, and edema were noted. At concentrations > 0.2 mg/ml severe curling of the tail and growth stunting occurred.

3. 5-Fluorouracil

5-Fluorouracil was strongly teratogenic to both Xenopus and Pimephales. Xenopus were affected at lower concentrations as with SA. Both demonstrated similar malformations and resulted in especially severe and stunted embryos at high concentrations.

The 120-hr EC50 (malformation) for Xenopus embryos exposed to FLU ranged from 0.06 to 1.00 mg/ml. The 120-hr LC50 ranged from 0.55 to 0.62 mg/ml. The resulting TI was 5.4-11.3 and the MCIG ranged from < 0.05 to 0.25 mg/ml. Multiple malformations consisting of gut, facial, eye, brain, heart and spinal malformations were common in concentrations > 0.02 mg/ml. At concentrations ranging from 0.1 to 0.3 mg/ml similar multiple malformations occurred in greater severity along with stunting, edema and blistering. At concentrations > 0.5 mg/ml embryos were so severely stunted that vitality was difficult to determine.

The 120-hr EC50 (malformation) for fathead minnow embryos exposed to FLU ranged from 0.15 to 0.77 mg/ml. The 120-hr LC50 ranged from 1.33 to 4.67 mg/ml. The resulting TI was 6.1-8.9 and the MCIG ranged from 0.08 to 0.10 mg/ml. The most common malformations induced by FLU in fish were spinal kinking, reduced eye size, and head malformations at concentrations > 0.1 mg/ml. At concentrations > 0.3 the severity of brain, eye, blistering, and spinal kinking increased. At concentrations greater than 0.5 mg/ml embryos were very stunted with heart malformations. (Plates 56-58)

These tests suggest that Xenopus laevis and fathead minnows respond with similar malformations when exposed to sodium acetate and 5-fluorouracil. Xenopus is affected at lower concentrations by SA and FLU. Caffeine was more teratogenic to fathead minnows and resulted in more severe malformations than Xenopus. Fish were malformed at lower concentrations but survived to higher concentrations than Xenopus when exposed to CAF. Further tests are planned using ^{14}C labeled material and liquid scintillation counting of whole fish and frog residue in order to related uptake to amount of mortality and malformation observed.

Table 82. 5-Fluorouracil

Concentration-Response Curve 120 hr. exposure

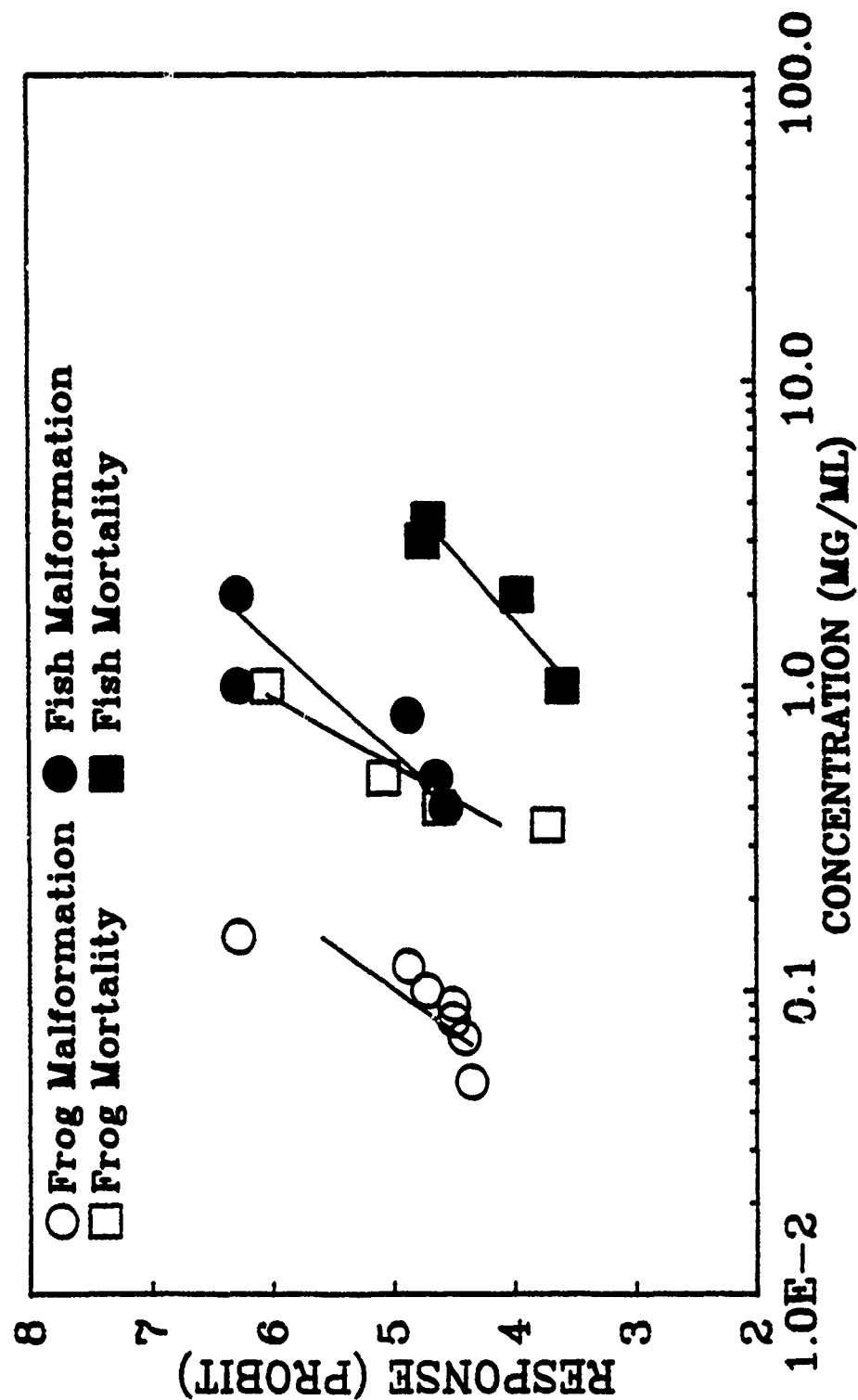


Table 83.

5-Fluorouracil

Growth Curve 120 hr. exposure

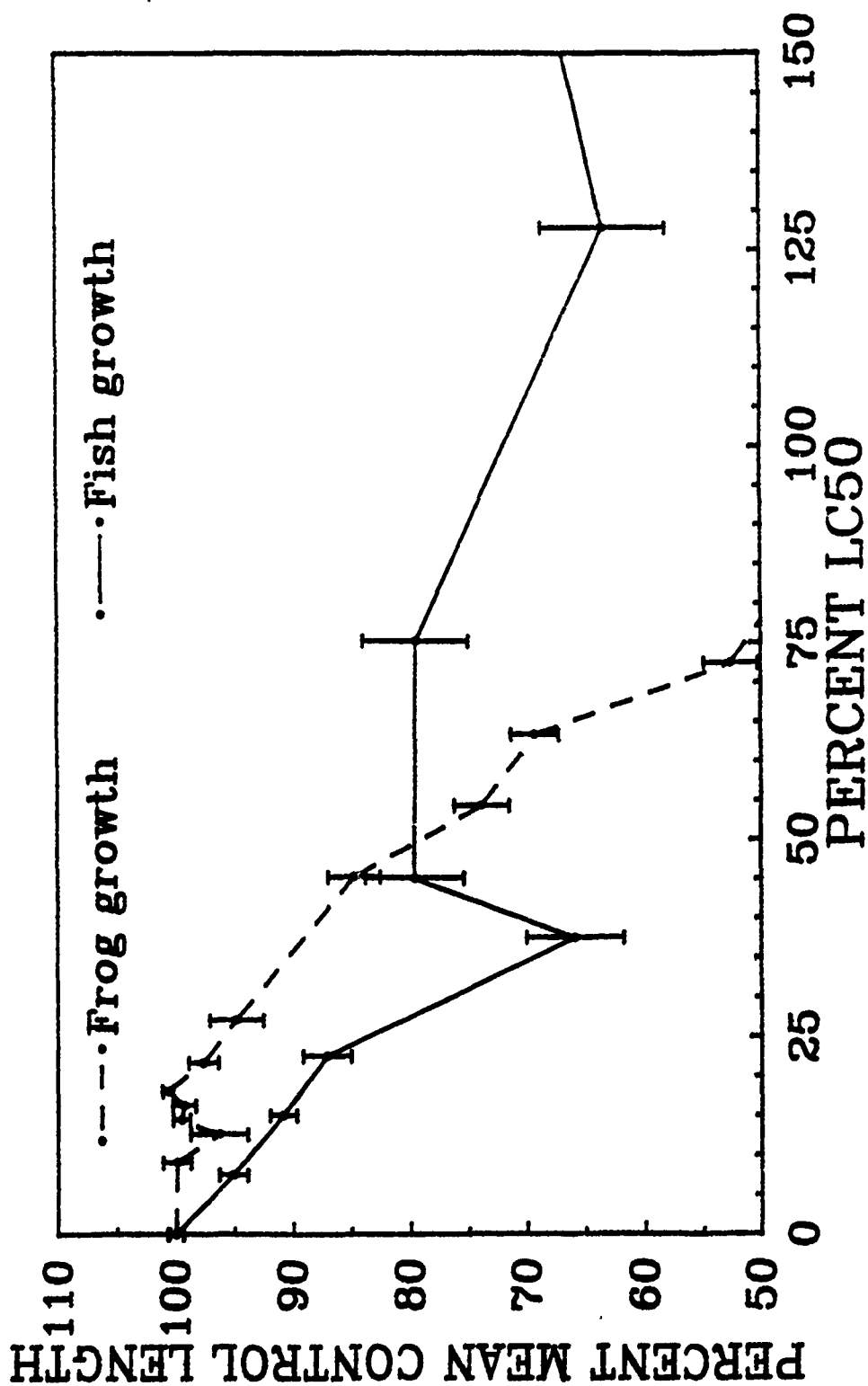


Table 84.

Sodium Acetate

Concentration-Response Curve 120 hr. exposure

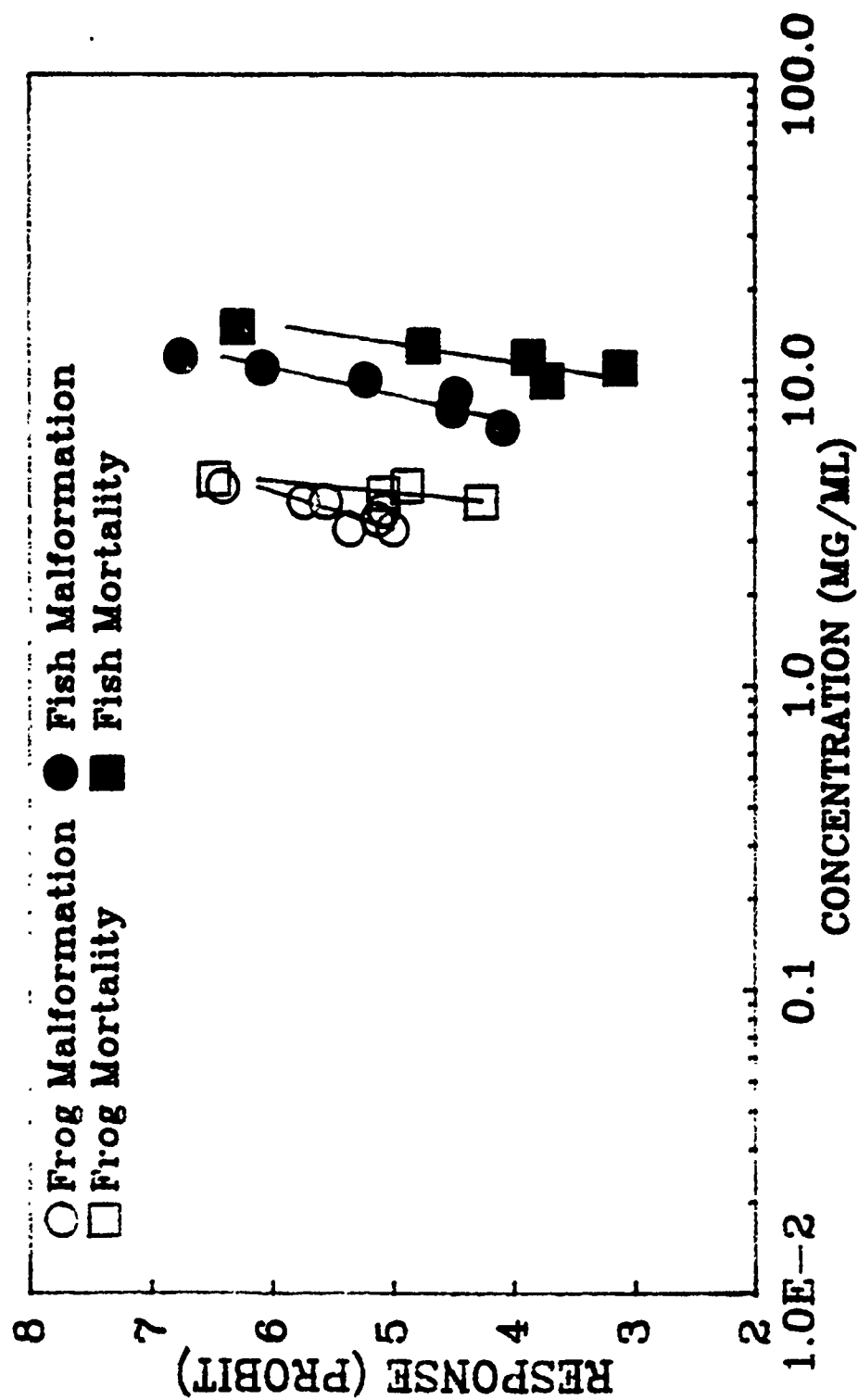


Table 85

Sodium Acetate

Growth Curve 120 hr. exposure

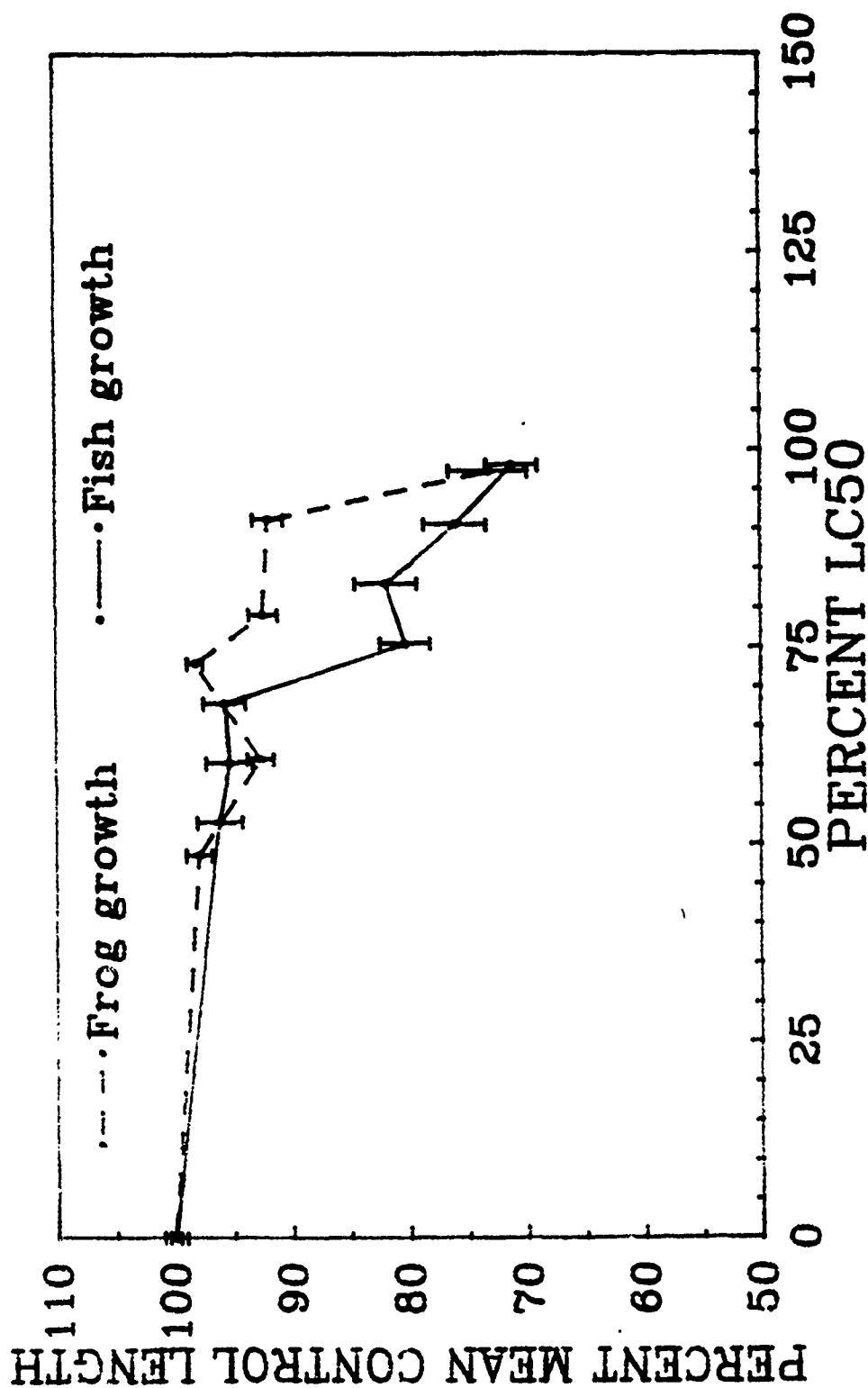


Table 86.

Caffeine

Concentration-Response Curve 120 hr. exposure

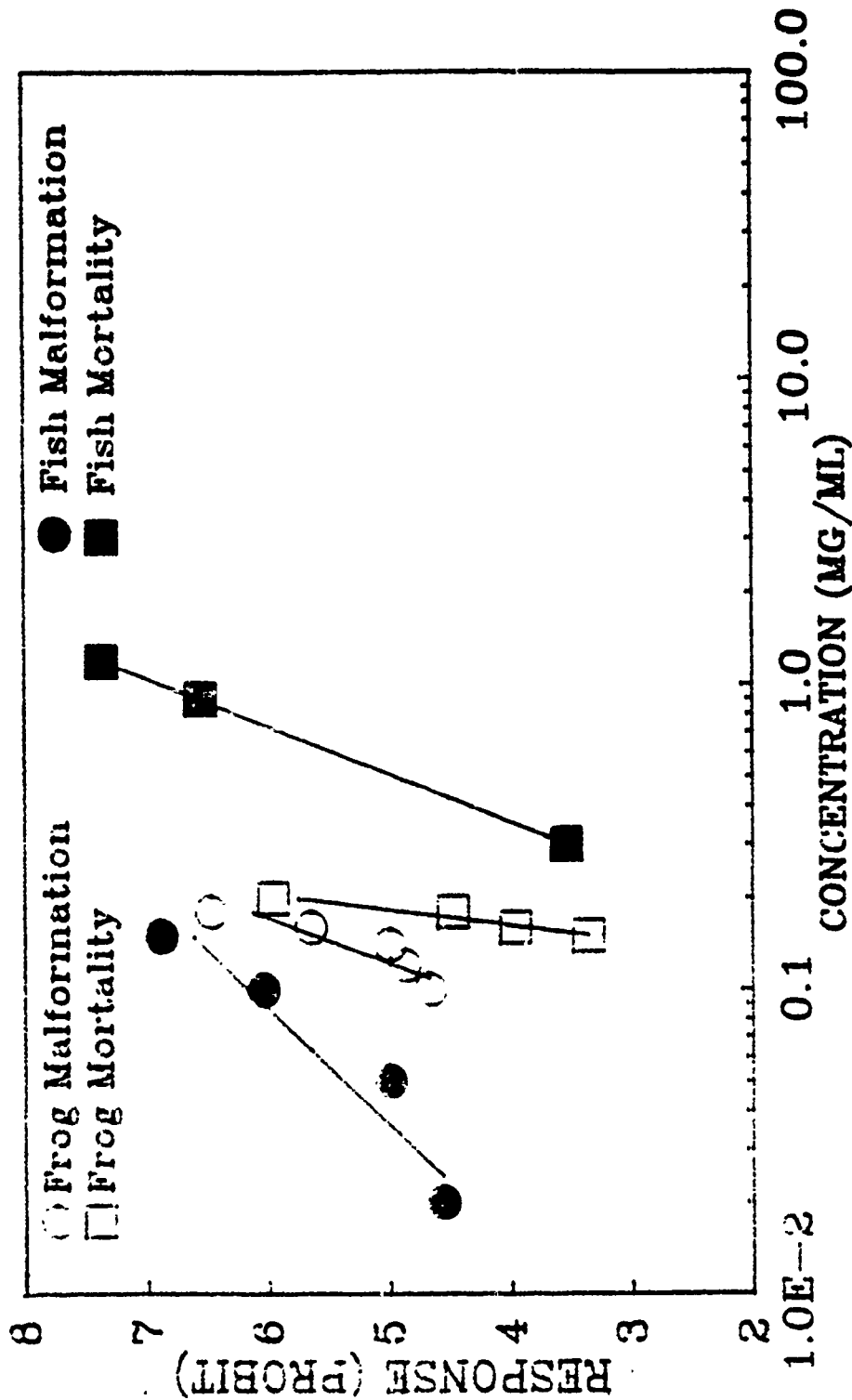
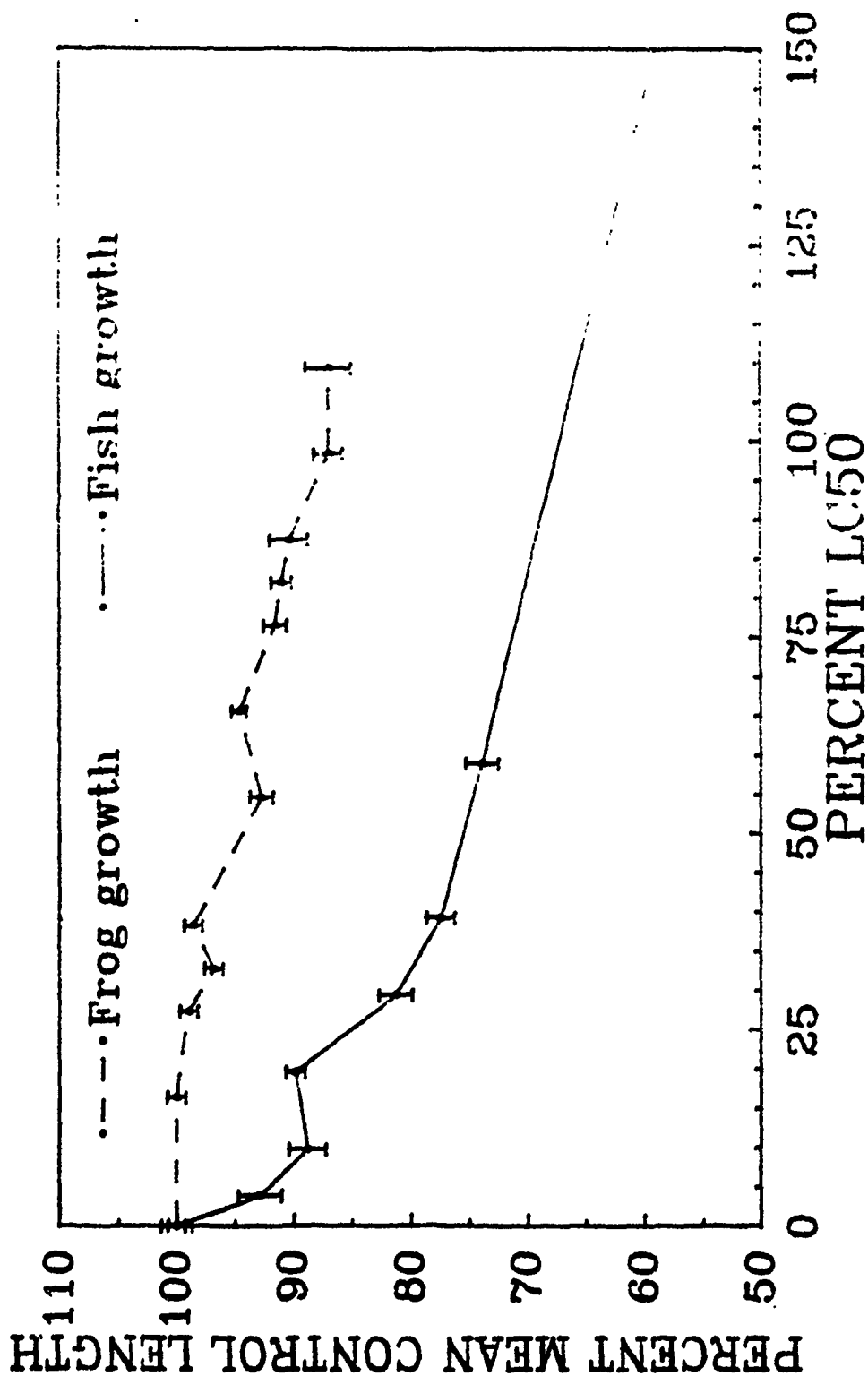


Table 87.

Caffeine

Growth Curve 120 hr. exposure



| Compound | Mean ^a LC50 | Mean ^a EC50 | Mean ^a H | Mean ^b MCIG | Mean ^b MCIG |
|--------------------------------|---------------------------|---------------------------|------------------------|---------------------------|---------------------------|
| Sodium Acetate CAS 127-09-3 | Frog 4.3 | 3.3 | 1.3 | 2.5 | 58 |
| | Fish 13.4 | 9.1 | 1.5 | 7.5 | 56 |
| Caffeine CAS 58-08-2 | Frog 0.2 | 0.1 | 1.8 | 0.2 | 100 |
| | Fish 0.6 | 0.07 | 2.6 | <0.02 | 3 |
| 5-Fluorouracil CAS 51-21-8 | Frog 0.5 | 0.1 | 5.5 | 0.1 | 20 |
| | Fish 3.0 | 0.5 | 7.5 | <0.1 | 3 |

^a All values are expressed in mg/ml, 95% confidence intervals were calculated but are not shown here

^b Minimum Concentration to Inhibit Growth as mg/L

^c Minimum Concentration to Inhibit Growth as a percent of LC50



Plate 50. The Effects of Different Concentrations of Sodium Acetate on *Xenopus laevis*. Embryos were exposed for 120-h. A side is view presented to show that only minor malformations occur. From top to bottom: Control, 3 mg/ml, 4 mg/ml and 5 mg/ml.

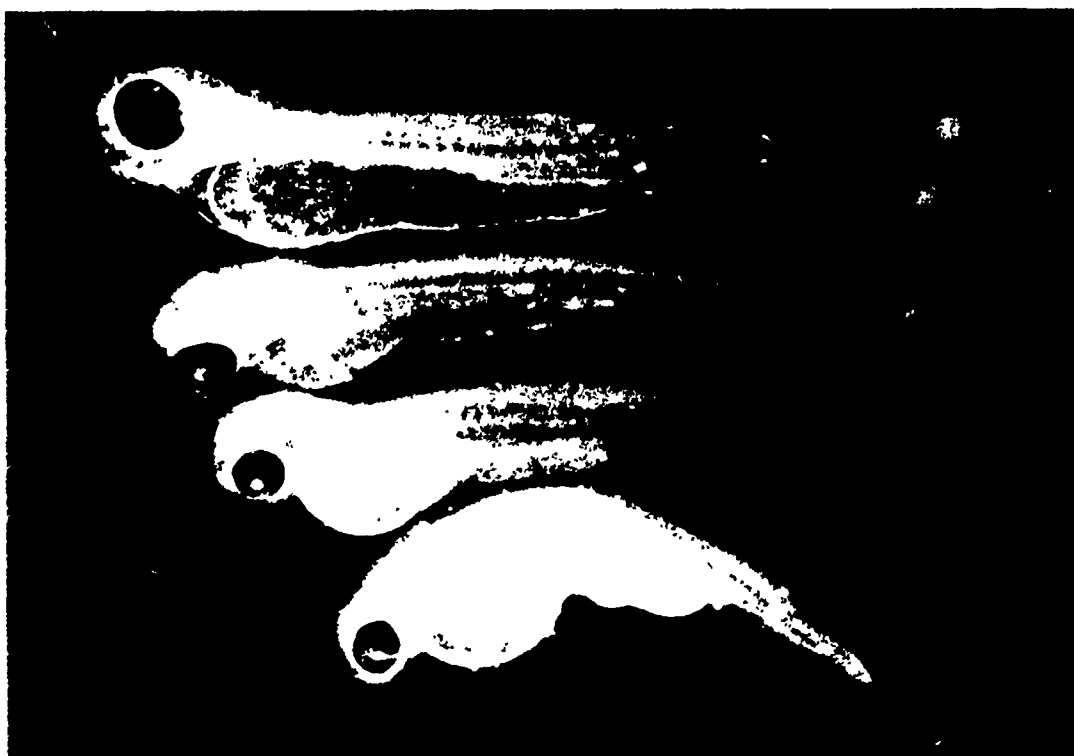


Plate 51. Effects of Different Concentrations of Sodium Acetate on *Pimephales promelas* Development. Fry were exposed for a length of 120-h. This side view presents the most common malformations seen in the fish, spinal kinking and stunting. From top to bottom: Control, 8.0 mg/ml, 11 mg/ml and 15 mg/ml.



Plate 52. The Comparative Effects of Different Concentrations of Sodium Acetate on *Xenopus laevis* and *Pimephales promelas*. Larvae were exposed to SA for 120-h. A side is view presented to show relative similarity in the malformations. From top to bottom: *Xenopus* control and high concentration. *Pimephales* control and high concentration.

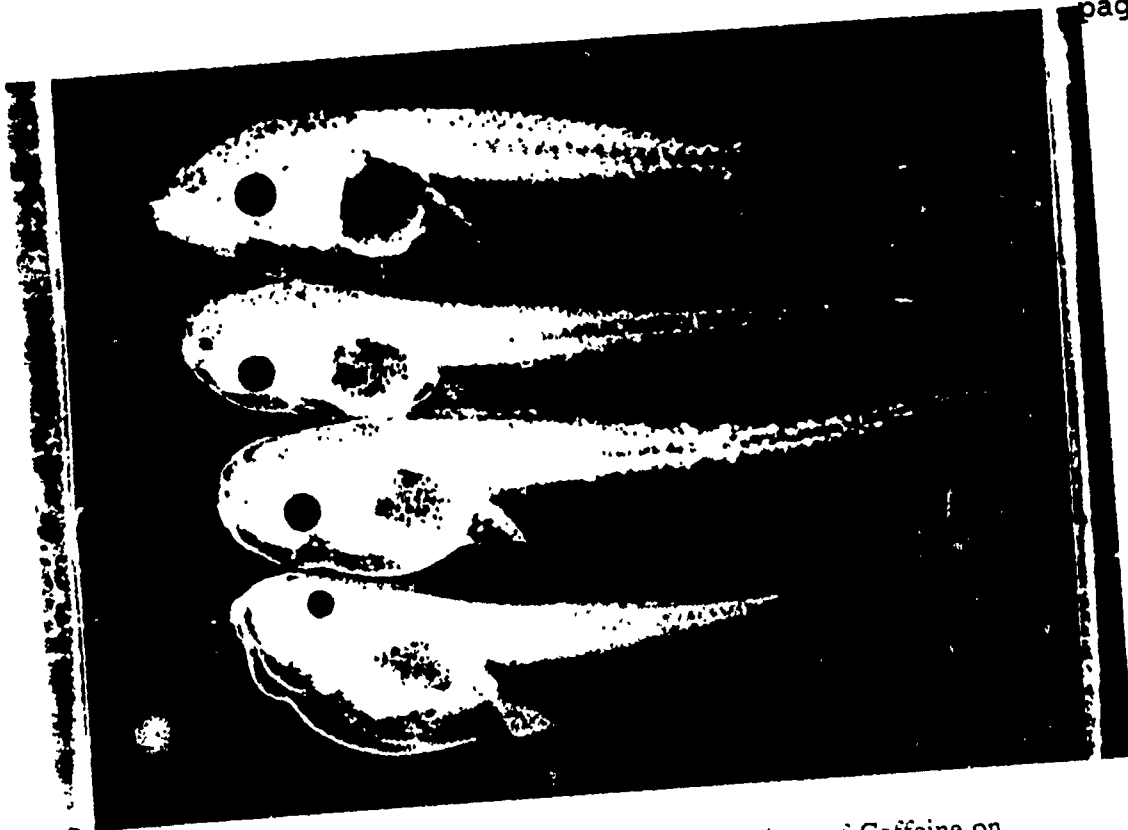


Plate 53. The Effects of Different Concentrations of Caffeine on *Xenopus laevis*. Embryos were exposed for 120-h. A side is view presented to show the moderate teratogenic effects: spinal kinking, improper gut coils and facial malformation. From top to bottom: Control, 0.12, 0.16 and 0.20 mg/ml.

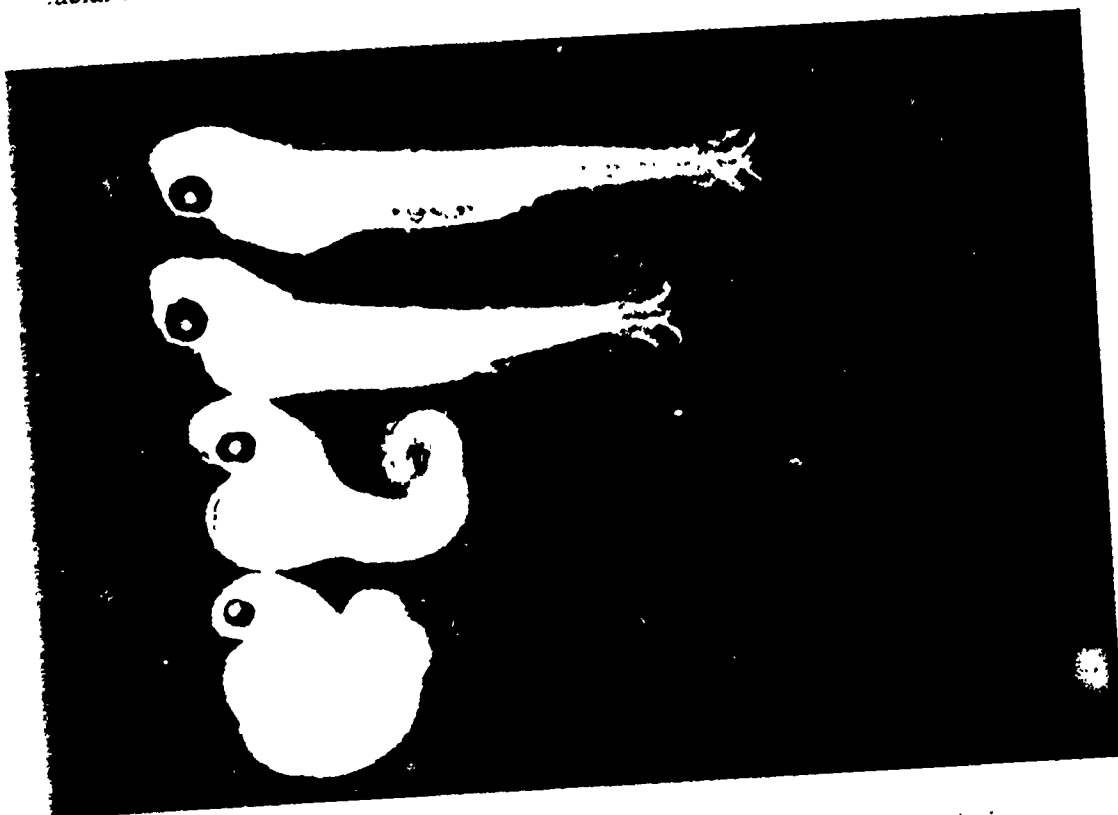


Plate 54. Effects of Different Concentrations of Caffeine on *Pimephales promelas* Development. Fry were exposed for a length of 120-h. This side view shows strong teratogenic effects of CAF on the fish. From top to bottom: Control, 0.05, 0.30 and 0.70 mg ml.

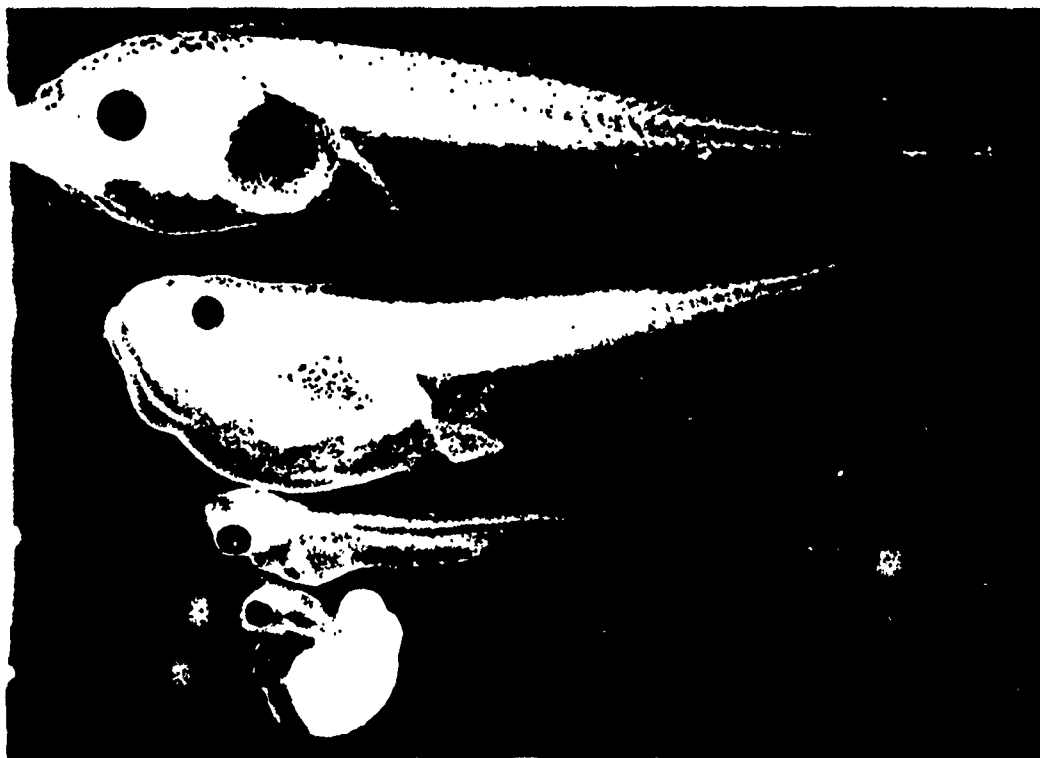


Plate 55. The Comparative Effects of Different Concentrations of Caffeine on *Xenopus laevis* and *Pimephales promelas*. Larvae were exposed to CAF for 120-h. A side view is presented to show differences in teratogenic intensity in the malformations. From top to bottom: *Xenopus* control and high concentration, *Pimephales* control and high concentration.



Plate 56. The Effects of Different Concentrations of 5-Fluorouracil on *Xenopus laevis*. Embryos were exposed for 120-h. A side is view presented to show that high degrees of malformations occur with FLU. From top to bottom: Control, 0.10 mg/ml, 0.25 mg/ml and 0.50 mg/ml.

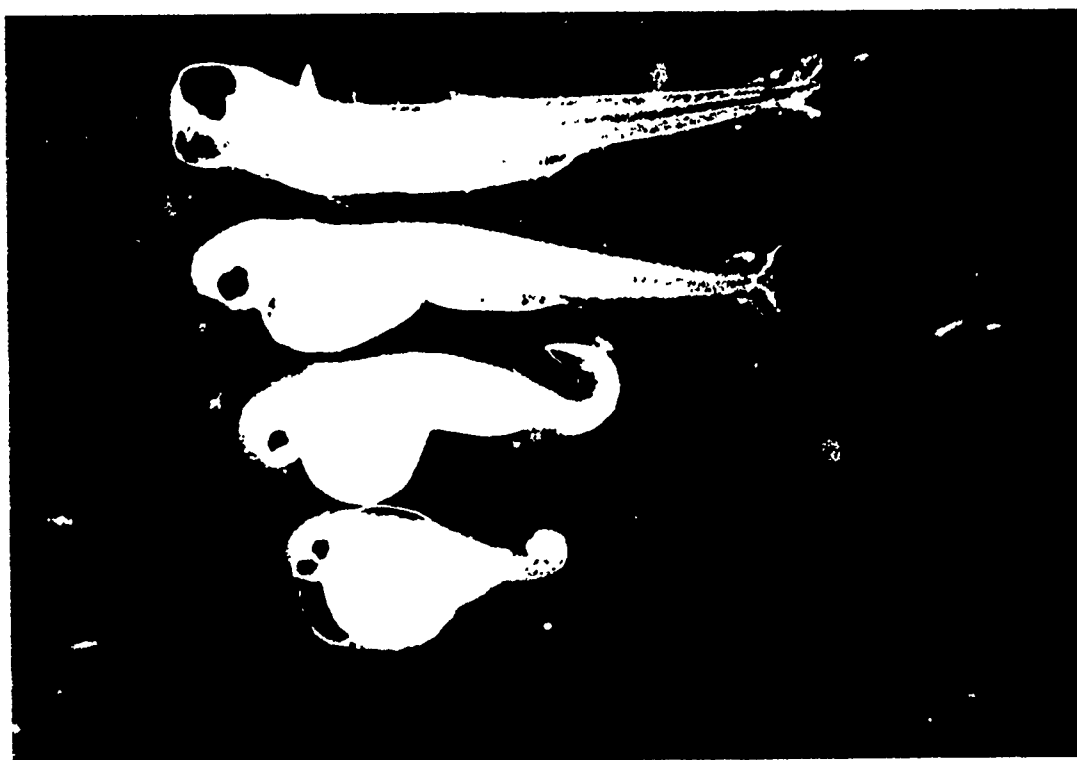


Plate 57. Effects of Different Concentrations of 5-Fluorouracil on *Pimephales promelas* Development. Fry were exposed for a length of 120-h. This side view presents the most common malformations seen, spinal kinking reduced eye size and head malformation. From top to bottom: Control, 0.10 mg/ml, 1.0 mg/ml and 2.0 mg/ml.

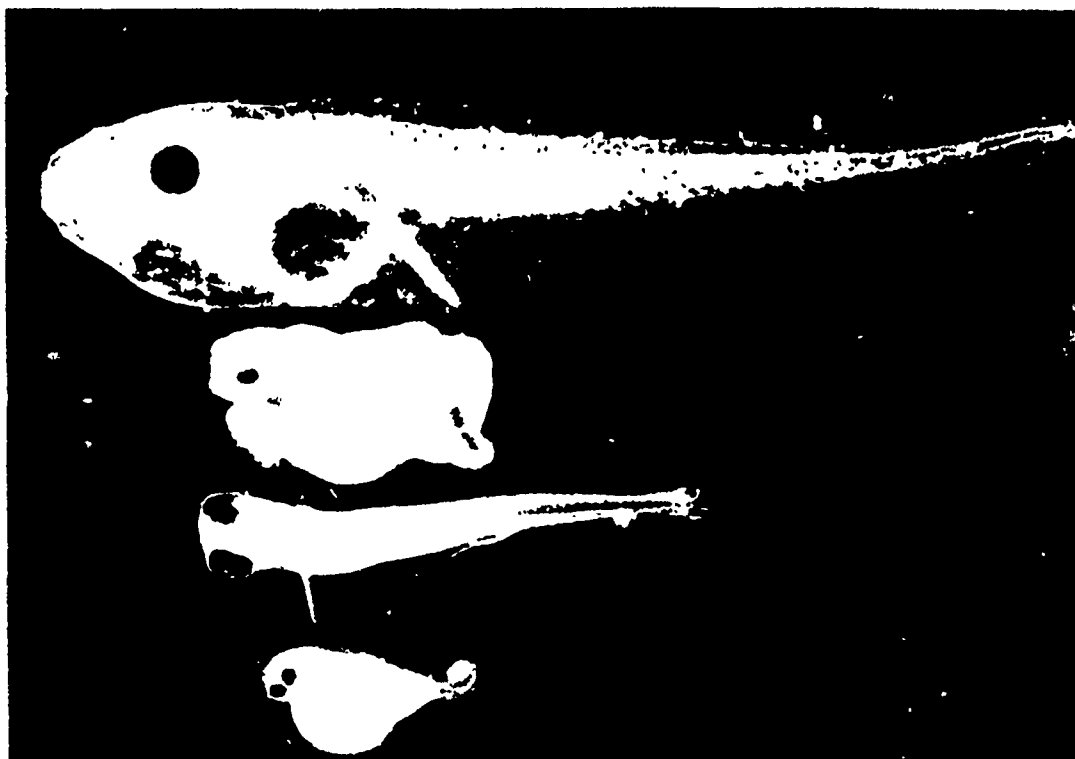


Plate 58. The Comparative Effects of Different Concentrations of 5-Fluorouracil on *Xenopus laevis* and *Pimephales promelas*. Larve were exposed to FLU for 120-h. A side is view presented to show similarity in the degree of malformations. From top to bottom: *Xenopus* control and high concentration, *Pimephales* control and high concentration .

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John A. Bantle
Oklahoma State University
Zoology Dept., 430 ISW
Stillwater, OK, 74078
(405) 744-9683
Thomas D. Sabourin
Environmental Sciences
Department
Battelle Columbus Division
505 King Ave.
Columbus, Ohio, 43201
(614) 424-7180

PROPOSED
NEW STANDARD GUIDE FOR CONDUCTING THE FROG, EMBRYO
TERATOGENESIS ASSAY-XENOPUS (FETAX)¹

1. Scope

1.1 This standard describes procedures for obtaining laboratory data concerning the developmental toxicity of a test material. The test utilizes embryos of the South African clawed frog, Xenopus laevis and is called FETAX (Frog Embryo Teratogenesis Assay- Xenopus)(1). Some of these procedures will be useful for conducting developmental toxicity tests with other species of frogs although numerous modifications might be necessary. A list of alternative anurans is presented in Appendix X1.

1.2 A renewal exposure regimen and the collection of the required mortality, malformation and growth-inhibition data are described. Special needs or circumstances might require

¹This standard is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E-47.01 on Aquatic Toxicology.

different types of exposure and data concerning other effects. Some of these modifications are listed in Appendix X2 although other modifications might also be necessary. Whenever these procedures are altered or other species used, the results of tests might not be comparable between modified and unmodified procedures. Any test that is conducted using modified procedures should be reported as having deviated from the standard guide.

1.3 These procedures are applicable to all chemicals either individually or in formulations, commercial products or mixtures that can be measured accurately at the necessary concentrations in water. With appropriate modification these procedures can be used to conduct tests on temperature, dissolved oxygen, pH, physical agents and on materials such as aqueous effluents (see Standard E 1192), leachates, aqueous extracts of water-insoluble materials, particulate matter, sediment and surface waters.

1.4 This standard may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the investigator using this standard to consult and establish appropriate safety and health practices and to determine the applicability of regulatory limitations prior to use.

1.5 This standard is arranged as follows:

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X1. List of Alternative species

X2. Additional endpoints and alternative exposures

2. Referenced Documents

2.1 ASTM Standards

E 380 Standard for Metric Practice²

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians

E 943 Definition of Terms Relating to Biological Effects and Environmental Fate.

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and their Uses.

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates and Amphibians

D 1193 Specification for Reagent Water.⁴

² Annual Book of ASTM Standards, Vol. 14.02; excerpts in gray pages of Vol. 11.04.

³ Annual Book of ASTM Standards, Vol. 11.04

⁴ Annual Book of ASTM Standards, Vol. 11.01

3. Terminology

3.1 The words "must," "should," "may," "can," and "might," have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 A developmental toxicant is a test material that affects any developmental process. Therefore, a developmental toxicant affects embryo mortality and malformation, and causes growth inhibition. A teratogen is a test material that causes abnormal morphogenesis (malformation). The Teratogenic Index or TI is a measure of developmental hazard (1). TI values higher than 1.5 signify a larger separation of the mortality and malformation concentration ranges and, therefore, a greater potential for all embryos to be malformed in the absence of

significant embryo mortality. The TI is defined as the 96-hr LC50 divided by the 96-hr EC50 (malformation).

3.3 For definitions of other terms used in this standard, refer to Standards E 729, E 943, and E 1023. For an explanation of units and symbols, refer to Standard E 380.

4. Summary of Standard

4.1 In FETAX, a range-finding and three definitive tests are performed on each test material. A control in which no test material has been added is used to provide 1) a measure of the acceptability of the test by indicating the quality of embryos and the suitability of the FETAX solution, test conditions and handling procedures and 2) a basis for interpreting data from other treatments. Each definitive test consists of several different concentrations of the test material with two replicates of each concentration. Each of the three definitive tests is conducted using embryos from a different male-female pair of Xenopus laevis. A reference toxicant (6-aminonicotinamide) should be used as a quality control measure. The 96-hr LC50 and 96-hr EC50 (malformation) are determined by probit analysis and the TI (Teratogenic Index) is calculated by dividing the LC50 by the EC50. Growth inhibition is determined by measuring the head-tail length of each embryo and determining whether growth at a particular concentration is significantly different from that in the control. Other useful data can be collected (eg. pigmentation, locomotion and hatchability) to expand the utility of the test.

5. Significance and Use

5.1 FETAX is a rapid test for identifying developmental toxicants. Data may be extrapolated to other species including mammals. FETAX might be used to prioritize samples for further tests which use mammals. Validation studies using compounds with known mammalian and/or human developmental toxicity suggest that the predictive accuracy will approach or exceed 85% (2). The accuracy rate compares favorably with other currently available "in vitro teratogenesis screening assays" (3). Any assay employing cells, parts of embryos, or whole embryos other than in vivo mammalian embryos is considered to be an in vitro assay.

5.2 It is important to measure developmental toxicity because embryo mortality, malformation and growth inhibition can often occur at concentrations far less than those required to affect adult organisms.

5.3 Because of the sensitivity of embryonic and early life stages, FETAX provides information that might be useful in estimating the chronic toxicity of a test material to aquatic organisms.

5.4 Results from FETAX might be useful when deriving water quality criteria for aquatic organisms (4).

5.4 FETAX results might be useful for studying structure-activity relationships between test materials and for studying bioavailability.

6. Safety Precautions

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and safety glasses, and using pipets to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers and the use of fume hoods, should be taken when conducting tests on volatile materials. Information provided in Material Safety Data Sheets on toxicity to humans (5), recommended handling procedures (6), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (7) and with test materials that are, or are suspected of being, carcinogenic (8).

6.2 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

6.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a fume hood.

6.4 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

6.5 Because FETAX solution and test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help avoid electrical shocks.

7. Apparatus

7.1 Facilities for Maintaining and Breeding Xenopus- Adults should be kept in an animal room that is isolated from extraneous light which might interfere with a consistent photoperiod of 12-hr day/12-hr night. The role that circadian rhythm plays in Xenopus reproduction has not been investigated. A constant photoperiod is therefore recommended so that Xenopus can be bred year-round. Adults can be kept in large aquaria or in fiberglass or stainless steel raceways at densities of 4-6 per 1800 square cm of water surface area. The sides of tanks should be opaque and at least 30 cm high. The water depth should be between 7 and 14 cm. Water temperature for adults should be $23 \pm 3^\circ\text{C}$.

Two types of breeding aquaria have been used successfully. A five or ten gallon glass aquarium may be used if fitted with a 1 cm mesh suspended about 3 cm from the bottom of the aquarium so that deposited eggs will lie undisturbed on the bottom of the aquarium. Hardware cloth or other metal mesh must not be used. Nylon or plastic mesh is recommended. The sides of the breeding aquarium should be opaque and a bubbler fitted to oxygenate the water. The top of the aquarium should be covered with an opaque porous material such as a fiberglass furnace filter. Alternatively, an adequate breeding tank can be constructed from two plastic dish pans (at least 38 x 38 cm) stacked one in the other. The floor of the topmost pan is perforated. A corkborer can be used to create 1.5 cm holes for the eggs to fall through.

7.2 Facilities for Conducting PETAX- A constant temperature room or a suitable incubator is required although a photoperiod is unnecessary. The incubator must be capable of holding $24 \pm 2^\circ\text{C}$. Abnormal development will occur at temperatures greater than 26°C . Covered 60 mm glass Petri dishes should be used as test chambers except that disposable 55 mm polystyrene Petri dishes should be used if a substantial amount of the test material binds to glass but not to polystyrene. A binocular dissection microscope capable of magnifications up to 30 X is required to count and evaluate abnormal embryos. A simple darkroom enlarger is used to enlarge embryo images 2-3 times for head-tail length measurements. It is also possible to measure embryo length through the use of a map measurer or an ocular micrometer. However, the process is greatly facilitated by using a digitizer interfaced to a microcomputer. The microcomputer is also used in data analysis.

7.3 Construction Materials- Equipment and facilities that contact stock solutions, test solutions, or water in which embryos will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that would adversely affect embryo growth or development. Additionally, items that contact stock solutions or test solutions should be chosen to minimize sorption of most test materials from water. Glass, Type 316 stainless steel, nylon and fluorocarbon plastic should be used whenever possible to minimize dissolution, leaching and sorption. Rigid plastics may be used for holding, acclimation and in the water supply system but they should be

oaked for a week before use in water used for adult maintenance.

FETAX solution, stock solutions, or test solutions should not contact brass, copper, lead, galvanized metal, or natural rubber before or during the test. Items made of neoprene rubber or other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect either survival or growth of the embryos and larvae of the test species.

7.4 Cleaning- At the end of each test, all glass dishes and other glassware that are to be used again should be immediately emptied, rinsed with water and cleaned by the following procedure.

Glassware washing procedure.

1. Soak 15 min, and scrub with tissue culture compatible detergent in tap water.
2. Rinse twice with tap water.
3. Rinse once with fresh, dilute (10%, v/v) hydrochloric acid to remove scale, metals and bases.
4. Rinse twice with tap water.
5. Rinse once with full strength reagent-grade⁵ acetone to remove organic compounds.
6. Rinse well with hot tap water.
7. Rinse well with distilled water or FETAX solution.
8. Heat the glassware in an oven at 350°C for 3 hrs to drive off any residual acetone. Toxicity problems have occurred in past experiments when this glassware washing procedure was omitted.

⁵ "Reagent Chemicals, American Chemical Society Specifications", Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards", by Joseph Rosin, D. Van Nostrand Co., Inc, New York, NY, and the "United States Pharmacopeia."

7.5 Acceptability- Before FETAX is conducted in new test facilities it is desirable to conduct a "non-toxicant" test, in which all test chambers contain FETAX solution with no added test material. The embryos should grow, develop and survive in numbers consistent with an acceptable test (see Section 14.1). The magnitude of the chamber-to-chamber variation should be evaluated.

8. Water for Culturing Xenopus Adults.

8.1 Requirements- Besides being available in adequate supply, the water should (a) allow satisfactory survival and reproduction of the adults, (b) be of uniform quality, and (c) not unnecessarily affect results of the test.

8.2 Source-

8.2.1 Natural water is preferred for adult culture. It should be obtained from an uncontaminated source that provides uniform quality. The quality of water from a well or spring is usually more uniform than that of a surface water. If a surface water is used as a source of fresh water, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. Water temperature should be adjusted to 23±3°C before being used to culture adults.

8.2.2 Dechlorinated water can be used to culture adults as long as residual chlorine and its oxidants are reduced to levels that do not affect survival and reproduction. Dechlorinated water should only be used as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines (9). Fluorides can be removed by passage over activated alumina columns (10). In addition to residual chlorine, chloramines and fluoride, municipal drinking water often contains unacceptably high concentrations of copper, lead,

and zinc, and quality is often rather variable. Excessive concentrations of most metals can usually be removed with a chelating resin (11).

8.3 Treatment-

8.3.1 Water for culturing adults should be aerated by the use of air stones or surface aerators. Air used for aeration should be free of fumes, oil, and water. Compressed air supplies might be contaminated with oil or water containing rust or sludge. Some compressed air supplies might also have a high level of carbon monoxide. A low pressure blower will provide high quality air without the problems associated with a high pressure air supply as long as its air supply is uncontaminated. Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. However, it is not absolutely necessary to aerate the water for Xenopus adults.

8.3.2 Filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low and as a pretreatment before filtration through a finer filter. Organics may be removed by filtration through activated charcoal filtration. Charcoal filters should be changed on a monthly basis.

8.3.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (12) equipped with an

intensity meter and flow controls, or passed through a filter with a pore size of 0.45 μ m or less.

8.4 Characterization-

8.4.1 The following items should be measured at least quarterly: pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, bromide, beryllium, cadmium, chromium, copper, iron, lead, manganese, mercury, nickel, selenium, silver and zinc. For each method used the detection limit should be below (a) the concentration in the water or (b) the lowest concentration that has been shown to adversely affect the test species.

8.4.2 Physical and chemical limits on water: pH should be between 6.5 and 9 (13). The TOC should be less than 10 mg/L, while alkalinity and hardness both should be between 16 and 400 mg/L as CaCO_3 (14). Table 1 shows the recommended maximum concentrations for some contaminants that have often been found to be in excess concentration in laboratory water supplies. The values reported are one-tenth of the minimum concentration that inhibits growth. While these data are not indicative of the effect of long-term exposure of adults on reproductive success, they, nonetheless, serve as a guide for limiting adult exposure to these metals. The maximum quantity of the other contaminants listed in 8.4.1 should meet EPA freshwater chronic water quality criteria (13).

TABLE 1. Recommended Maximum Concentrations of Some Metals.

| Metal* | Recommended Maximum Concentration (μ g/L) |
|------------------------|--|
| Cadmium (2) | 10.0 |
| Lead (2) | 5.0 |
| Mercury (2) | 0.144 |
| Nickel (2) | 25.0 |
| Selenium (unpublished) | 140.0 |
| Zinc (2) | 70.0 |

*Tested in FETAX at 100 mg/L hardness as CaCO_3 . Values reported are one-tenth of the minimum concentration to inhibit growth.

9. FETAX Solution Water

9.1 Requirements- FETAX solution should be used for breeding and static or renewal assays. FETAX solution should also be used for flow-through experiments whenever possible. However, should the need for a large volume preclude the use of FETAX solution, then water conforming to the specifications listed in section 8 may be used. The water must allow embryonic growth at the same rate as FETAX solution and there should be no differences between control mortality and malformation rates.

9.2 Formulation- FETAX solution is composed of 625 mg NaCl, 96 mg NaHCO_3 , 30 mg KCl, 15 mg CaCl_2 , 60 mg $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and 75 mg MgSO_4 per liter of deionized or distilled water. The pH of the final solution should be 7.6-7.9. All chemicals should be reagent-grade⁵ or better. Deionized or distilled water must conform to Type I ASTM water (Standard D 1193).

10. Test Material

10.1 General- The test material should be reagent-grade⁵ or better unless a specific test involves an unknown complex mixture, formulation, commercial product, or technical-grade or use-grade material. Before a test is begun, the following should be known about the test material:

10.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1% of the material.

10.1.2 Solubility and stability in water.

10.1.3 Estimate of toxicity to humans.

10.1.4 Recommended handling procedures (see Section 6).

10.1.5 For unknown samples much of the information specified in 10.1.1-10.1.4 will be lacking, but the pH, hardness, alkalinity and conductivity of the sample should be measured.

10.2 Stock Solution-

10.2.1 In most cases the test material can be added directly to the FETAX solution in the Petri dishes but usually it is dissolved in a solvent to form a stock solution. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. Stock solutions should be prepared daily unless analytical data indicate the solution is stable with time. If the test material is subject to photolysis, the stock solution should be shielded from light.

10.2.2 Except possibly for tests on hydrolyzable, dizable, and reducible materials, the preferred solvent is FETAX solution. The minimum necessary amount of a strong acid base may be used in the preparation of an aqueous stock solution, but such acid or base might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, lithium or potassium salts of phenols or organic acids, and oxide or nitrate salts of metals, might affect the pH. The use of minimum necessary amount of a strong acid or base. Any adjustments of pH can send the test material through transition to affect changes in such properties as solubility or degree and type of dissociation. Prior to testing, as chemical and physical data as are available on the test material should be obtained and considered prior to making adjustments on pH adjustments.

If a solvent other than FETAX solution is used, its concentration in test solutions should be kept to a minimum and would be low enough that it does not affect Xenopus embryo growth and survival. Because of its low toxicity, low activity, and high ability to dissolve many organic chemicals, ethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as dimethyl sulfoxide and acetone also may be used. Concentrations of triethylene glycol, dimethyl sulfoxide and acetone in test solutions should be <1.6%, <1.1%, <1.1% v/v respectively. These concentrations have been found

not to cause any adverse effects in FETAX (15). At times, concentrations approaching 1% solvent are necessary to keep test materials in solution for FETAX. This is often the case when the assay is used in testing pure compounds for the purpose of comparing test results with mammalian data. If possible, it is desirable to perform the test using two different solvents and compare the results. This will help in identifying possible interactions between a solvent and test material.

Ethanol is not recommended because its teratogenic index (TI) in FETAX is around 1.4. Methanol has high toxicity in FETAX. Acetone might stimulate the growth of microorganisms and is quite volatile. If an organic solvent is used it should be reagent-grade⁵ or better. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions.

10.2.3 If a solvent other than dilution-water or FETAX solution is used, (a) at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test and (b) a dilution-water or FETAX solution control should be included in the test. If no solvent other than dilution-water or FETAX solution is used, then a dilution-water or FETAX solution control must be included in the test.

10.2.3.1 The concentration of solvent must be the same in all test solutions that contain test material and the solvent control must contain the same concentration of solvent.

10.2.3.2 If the test contains both a dilution-water or a FETAX-solution control and a solvent control, the mortality, malformation and growth inhibition should be compared using a two-tailed t-test. If a statistically significant difference in either mortality, malformation, or growth inhibition is detected between the two controls, only the solvent control may be used as the basis for comparison in the calculation of results.

10.2.3.3 If a solvent other than dilution-water or FETAX solution is used to prepare a stock solution, it might be desirable to conduct simultaneous tests using chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvents on results of the test.

11. Test Organisms

11.1 Species- FETAX is designed to use embryos of the South African clawed frog Xenopus laevis (Daudin). Information regarding the basic biology and development of this species has been reported by Deuchar (16,17). Appendix XI lists other North American species that can be used in situations where Xenopus cannot, although there will be differences in the rate of development and the method of inducing breeding. Many anurans only breed in a specific season during the year. The length of exposure might have to be altered to allow proper organogenesis.

11.2 Source- Adult frogs for breeding may be obtained from various supply houses or independent suppliers. Proven breeders should be requested from the supplier. Each animal should be thoroughly examined upon arrival for skin lesions or red patches on the ventral surfaces. Skin lesions are indicative of nematode infection while the red patches indicate Aeromonas infection. Care should be taken to ensure that only healthy, sexually mature frogs are placed in the colony.

11.3 Adults-

11.3.1 Selection- Xenopus males should be 7.5 to 10 cm in crown-rump length and at least two years of age. Males have dark arm pads on the underside of each forearm and lack cloacal lips. Females should be 10 to 12.5 cm in length and at least three years old. Females are always larger than males and easily identified by the presence of fleshy cloacal lips.

11.3.2 Diet- The minimum recommended diet for adults
ld be three feedings per week of ground adult beef liver.

Liquid multiple vitamins should be added to the ground beef
r. The concentration of vitamins is shown in Table 2.
entratons of vitamins from 0.05 to 0.075 cc/5 gm liver are
opriate. All food should be screened for the test material
he test material is present in the environment. All liver
must meet USDA standards for human consumption.

Table 2. Recommended Concentration of Vitamins*

| | |
|-------------------------------|------|
| Vitamin A, IU | 1500 |
| Vitamin D, IU | 400 |
| Vitamin E, IU | 5 |
| Vitamin C, mg | 35 |
| Thiamine, mg | 0.5 |
| Riboflavin, mg | 0.6 |
| Niacin, mg | 8 |
| Vitamin B ₆ , mg | 0.4 |
| Vitamin B ₁₂ , mcg | 2 |

*per ml of solution.

11.3.3 Temperature- Adults should kept at 23 \pm 3°C.

11.3.4 Circadian rhythm- Adults should kept on a 12-
ay/12-hr night cycle. The role that circadian rhythm plays
enopus reproduction has not been investigated. Thus, a
stent photoperiod should be maintained.

11.4 Breeding- Males and females are bred as a single pair.
frogs should be bred in the same water in which the test is
e conducted. To induce breeding the male and the female
ld receive 250-500 and 500-1000 IU, respectively, of human
ionic gonadotropin via injection into the dorsal lymph sac.

The hormone concentration should be 1000 IU per ml in sterile
0.9% NaCl. A 1 ml tuberculin syringe fitted with a 1/2" long,
26 gauge needle should be used to make the injection. Larger
bore needles might allow leakage of hormone from the injection
site. The amount of human chorionic gonadotropin injected
depends on the time of year and condition of the adults. Lower
doses are usually used in spring and higher doses in fall.
Amplexus normally ensues within 2-6 hr and egg deposition about
9-12 hr after injection. The eggs should be immediately
inspected for fertility and quality. The fertility rate should
be >75%. Eggs laid in "strings" or not perfectly round should
not be used because they develop abnormally.

11.5 Embryos-

11.5.1 Removal of Jelly Coat- Dejellying of embryos
should begin immediately following the end of egg laying.
Dejellying of embryos should be carried out by gentle swirling
for 1-3 minutes in a 2% w/v L-cysteine (CAS # 52-90-4) solution
prepared in FETAX solution. The cysteine solution should be
adjusted to pH 8.1 with 1N NaOH. The solution should be made up
immediately prior to use. Dejellying should be monitored
continuously and the process stopped just after all jelly is
removed. Care should be taken not to treat the embryos too long
because survival will be reduced.

11.5.2 Staging of Embryos- Nieuwkoop and Faber must be
used in all staging of embryos (18).

11.5.3 Embryo Selection- Normally cleaving embryos must be
selected for use in testing. The "Atlas of Abnormalities"

(available from John A. Bantle, Dept. of Zoology, 430 ISW, Oklahoma State University, Stillwater, OK, 74078) should be consulted in order to determine which embryos are normal. It is best to use two levels of selection. In double selection, normally cleaving embryos are first sorted into dishes containing fresh FETAX solution. After a short period during which cleavage continues, embryos are again sorted ensuring that only normal embryos are selected. Abnormal pigmentation should be viewed as an indicator of bad embryos. Either Nieuwkoop and Faber (18) or the "Atlas of Abnormalities" can be used as a reference to determine whether the cleavage pattern is normal. Midblastula (Stage 8) to early gastrula (Stage 11) must be used to start the test. Embryos chosen prior to Stage 8 might develop abnormal cleavage patterns later whereas embryos selected after Stage 11 have commenced organogenesis. A large bore blood bank Pasteur pipet can be used to transfer embryos at this stage without harm. The sorting should be done in 100 mm Petri dishes.

12. Procedure

12.1 Description- FETAX is a 96-hr renewal whole embryo assay that can be used to evaluate the developmental toxicity of a test material. Exposure is continuous throughout the test. For each concentration two dishes each containing 25 embryos and 10 ml of test solution are used. For each control, four dishes of 25 embryos each are used. Embryos must be randomly assigned to test dishes. Dishes must be randomly assigned to their positions in the incubator. In order to properly evaluate developmental toxicity, mortality, malformation, and growth-inhibition data must be collected. In most tests it will be possible to generate concentration-response curves for mortality, malformation and growth inhibition. The mortality and malformation concentration-response curves should then be used to estimate the concentration that would affect 50% of the exposed embryos. At least 90% of the FETAX-solution controls must have attained Stage 46 at 96 hrs (18).

12.2 Temperature and pH Requirements--

12.2.1 Temperature- $24 \pm 2^\circ\text{C}$ must be maintained throughout the 96-hr test. Temperatures higher than 26°C cause malformation whereas low temperatures prevent the controls from reaching Stage 46 in 96 hrs.

12.2.2 pH- The pH of the stock and test solutions should be 7.7 and must be between 6.5 and 9.0 (13). The pH of a control dish and the pH of the highest test concentration should be measured at the beginning of the test and every 24 hrs thereafter to determine if they have changed.

12.3 Beginning the Test-

12.3.1 Range-finding- A range-finding test is recommended. The range-finding test should consist of a series of at least seven concentrations that differ by a factor of ten. This should be adequate for delineating the concentrations needed to establish the definitive 96-hr LC50 and EC50 (malformation). The greater the similarity between the range-finding and definitive test, the more useful the range-finding test will be. Growth inhibition data are not collected from range-finding tests.

12.3.2 Definitive Tests- Three definitive tests should be conducted on each test material. Each definitive test comprises a concentration series that will generate concentration-response data adequate for determining the 96-hr LC50 and EC50 (malformation) (see Section 12.3.3; 12.6.1; 12.6.2). A definitive test consists of a geometric series of concentrations that varies by a factor of about two. Because it is necessary to acquire data on mortality, malformation and growth inhibition, the concentration series needs to be adjusted so the expected 96-hr LC50, 96-EC50 (malformation) and the minimum concentration needed to inhibit growth (MCIG). To insure an adequate supply of normal embryos for each definitive test, three mating pairs should be induced and clutches harvested separately. Embryos should be sorted to assure viability prior to testing. Each test uses early embryos derived from a single mating pair; if the controls from a particular mating pair indicate a problem with fertility or

viability of early embryos, the test will be unacceptable for that particular clutch. Each individual test will yield data that will be used to generate concentration-response curves for mortality, malformation, and growth inhibition.

12.3.3 Experimental Dilutions- Each test should consist of at least five concentrations for determining concentration-response curves for both mortality and malformation. At least three concentrations should be within 16-84% effect on the mortality and malformation concentration-response curves. Each test should consist of two dishes for each concentration of test material and four dishes for each control.

Each test should be performed with embryos derived from a single mating pair regardless of the number of replicate dishes. The selection of experimental design and statistical methods required to evaluate mortality, malformation and growth-inhibition data should consider the type of compound or chemical mixture being evaluated and the limitations that sample or time availability might imply as far as appropriate statistical techniques (19).

12.3.4 Reference Toxicant- For a positive control or reference toxicant, 6-aminonicotinamide presents a mortality and malformation data base convenient for reference purposes. Commercial sources for the 6-aminonicotinamide (CAS #329-89-5); formula weight, 137.14) should specify the physicochemical data and the purity for the compound which assure its being comparable to that readily available to other laboratories

(e.g., UV spectroscopic characterization: at A_{257} and pH 1.8, 6-aminonicotinamide has an extinction coefficient of 13.9 mM and an absorbance ratio (A_{257}/A_{302}) of 2.20). The purity should be >99%. From this published data base for 6-aminonicotinamide, the 96-hr LC50 is 2500 mg/L (95% CI=2350-2650) and the 96-hr EC50 for malformation is 5.5 mg/L (95% CI=3.9-6.9), or a Teratogenic Index of 455 (20). For each test, the positive control consists of exposing two dishes of 25 embryos each to 2500 mg 6-aminonicotinamide/L and two dishes of 25 embryos each to 5.5 mg 6-aminonicotinamide/L. The mortality and malformation observed should be between 40 and 60%. For example, at 2500 mg/L, 20 to 30 of the 50 embryos should have died by 96 hr. Only those biological responses related to mortality and malformation are considered in this analysis; growth inhibition is not considered in regard to responses to 6-aminonicotinamide. If results with the reference toxicant are inconsistent or at variance with previous experience, the source of the difference and the influence on test results should be considered.

12.4 Renewal- The renewal procedure should be used for the standard FETAX test. The renewal procedure entails fresh replacement of test material every 24 hr during the test. Just prior to this change it is advisable to measure the pH of the control and the highest test solution in order to determine if significant changes occurred. Renewal should be accomplished by removing the test solution with a Pasteur pipet. The orifice of the Pasteur pipet should be enlarged and fire-polished to accommodate embryos without damage in case the embryos are

accidentally picked up. This procedure should proceed quickly in order to minimize embryo desiccation. This is the standard procedure for FETAX but two other variants are allowed as described in the Appendix X2. Variations to the renewal procedure must be reported.

12.5 Duration of the Test- The standard exposure time for FETAX is 96 hr and the attainment of Stage 46 in controls. Deviations from this standard exposure time must be reported as deviating from standard FETAX conditions.

12.6 Biological Data-

12.6.1 Mortality- Dead embryos must be removed at the end of each 24-hr period during the 96-hr test at the time solutions are changed. If dead embryos are not removed, then microbial growth can occur that might kill live embryos. Death at 24 hr (Stage 27) is ascertained by the embryo's skin pigmentation, structural integrity, and irritability. At 48 hr (Stage 35), 72 hr (Stage 42) and 96 hr (Stage 46) the lack of heartbeat serves as an unambiguous sign of death. At 96 hr of exposure or Stage 46 of controls, the number dead is recorded. Dead embryos must be removed and the remaining live embryos fixed in 3% formalin.

12.6.2 Malformation- Malformations must be recorded at the end of 96 hr. The "Atlas of Abnormalities" should be used in scoring malformations, particularly slight malformations. Embryos exposed to the test material should also be compared to appropriate controls. The number of

13. Analytical Methodology

13.1 The methods used to analyze test solutions might determine the usefulness of the test results if the results are based on measured concentrations. For example, if the analytical method measures any impurities or reaction or degradation products along with the parent test material, results can be calculated only for the whole group of materials, and not for the parent material by itself, unless it is demonstrated that such impurities and products are not present.

13.2 If samples of stock solutions or test solutions cannot be analyzed immediately, they should be handled and stored appropriately (21) to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

13.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist, methods should be obtained from other reliable sources (22).

14. Acceptability of Test

14.1 A test using embryos from a single mating pair should be considered unacceptable if one or more of the following occurred.

14.1.1 Hardware cloth or metal mesh was used as a support in the breeding aquarium.

14.1.2 In the controls, either (or both) the mean survival is <90% or the mean malformation in embryos is >7%.

14.1.3 If 90% of the FETAX-solution-only controls do not reach Stage 46 by the end of 96 hr. The primary cause of control embryos not reaching Stage 46 is low temperature (see Section 12.2.1).

14.1.4 If dilution water was used in the test, it did not allow embryonic growth at the same rate as FETAX solution.

14.1.5 The deionized or distilled water does not conform to Type I ASTM standard.

14.1.6 A required dilution-water or FETAX solution control or solvent control was not included in the test.

14.1.7 The concentration of solvent was not the same in all treatments, except for a dilution-water or FETAX-solution control.

14.1.8 Staging of embryos was performed using a reference other than Nieuwkoop and Faber (18).

14.1.9 The test either was started with less than Stage 8 blastulae or greater than Stage 11 gastrulae.

14.1.10 All Petri dishes were not physically identical throughout the test.

14.1.11 Petri dishes were not randomly assigned to their positions in the incubator.

14.1.12 The embryos were not randomly assigned to the petri dishes.

14.1.13 Required data concerning mortality, malformation and growth were not collected.

14.1.14 The pH of the test solution was <6.5 or >9.0 in the control or highest test concentration.

14.1.15 Dead embryos were not removed after each 24-hr (± 2 hr) interval.

14.1.16 Consistently deviating from the temperature limits as stated in 12.2.1. A short-term deviation of more than $\pm 2^\circ\text{C}$ might be inconsequential.

14.1.17 If the reference toxicant was used, there were <20 or >30 of 50 embryos killed in the 2500 mg 6-aminonicotinamide/L positive control and/or <20 or >30 of 50 embryos malformed at 5.5 mg 6-aminonicotinamide/L.

15. Documentation

15.1 The record of the results of an acceptable PETAX should include the following information either directly or by reference to existing publications.

15.1.1 Name of test material, investigator(s) name, location of laboratory, and dates of initiation and termination of test.

15.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used. For some complex environmental mixtures a great deal of this information might be lacking.

15.1.3 If a dilution water other than PETAX solution is used, its chemical characteristics and a description of any pretreatment.

15.1.4 Recent analyses of PETAX solution and adult culture water.

15.1.5 pH measurements of control and of the highest test concentrations at the end of each 24-hr time period. Available data on sample hardness, alkalinity, conductivity, total organic carbon (TOC), concentration of dissolved oxygen and metal content.

15.1.6 The mortality, malformation rates and the mean embryo length at 96 hr in the dilution-water, PETAX solution or solvent control.

15.1.1.7 The mortality and malformation results obtained for the 6-aminonicotinamide positive control. If a full concentration-response curve was performed, then the 96-hr LC50, the 96-hr EC50(malformation) and their confidence limits should be reported.

15.1.1.8 The 96-hr LC50, the 96-hr EC50(malformation), the TI (96-hr LC50/96-hr EC50 (malformation)) and the minimum concentration to inhibit growth (MCIG) for each definitive test. The geometric means of these values and their 95% confidence limits. Concentration-response curves for mortality, malformation and growth inhibition may be provided.

15.1.1.9 A table for each test that lists the % mortality, % malformation and the head-tail length at each concentration tested.

15.1.1.10 The names of the statistical tests employed, the alpha-levels of the tests and some measure of the variability of the hypothesis tested.

15.1.1.11 The types, frequency and severity of malformations. The types of malformations and their severity might differ over the different concentrations tested. It might be best to define ranges of concentrations tested and create a summary table that lists the malformations that occurred in each concentration range.

15.1.1.12 Any deviations from standard FETAX (see Appendices XI and X2).

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Appendix

XI. List of Alternative Species.

XI.1 Use of alternative species- Although PETAX was designed expressly for the use of Xenopus laevis, it might be necessary to use endemic species when required by regulations or other considerations. Users are cautioned that many endemic species of frogs are threatened by pollution and habitat loss and the user should carefully consider the environmental consequences of large scale collection of local anuran species. Deviations from standard procedures must be reported (see Section 15) and it will be difficult to compare data between standard PETAX and data derived using an alternative species.

XI.2 Recommended anurans- Members of the family Ranidae (e.g., Rana pipiens) and Bufonidae (e.g., Bufo fowleri) might be best suited for PETAX, because the number of eggs and/or the seasonal availability are more limited for other species. Seasonal availability can be extended by 2-3 months using human chorionic gonadotropin injection. Rana catesbeiana and Bufo americanus are likely as well suited as Rana pipiens and Bufo fowleri (Table 3). High egg production, geographical range, short hatching periods, and other factors would indicate that these four species could serve as alternatives. Comparative sensitivities to inorganic mercury have been reported for some of these species (23). These studies have reported a range in sensitivity to inorganic mercury which should be taken into account when comparing data with other amphibian species.

Appendix

X2. Additional Data and Alternative Exposures

X2.1 Additional Data- Other types of data can be collected in FETAX that increases its versatility. The types of data listed below represent some that have been collected in past experiments. In the case of pigmentation and locomotion, scoring is subjective.

X2.1.1 Pigmentation- Collecting data on pigmentation might be useful for measuring neural damage because it is thought that the size of the pigment patches is under nervous control. Agents that affect these nerves cause smaller pigment patches and the overall color of the 96-hr larvae will pale. Comparison to the standard "Atlas of Abnormalities" and suitable controls must be made in order to determine abnormal pigmentation. Other causes of depigmentation are possible including loss of melanin production. A concentration-response curve can be generated and an EC50 (pigmentation) determined.

X2.1.2 Locomotion- Collecting locomotion data is potentially useful in measuring specific neural or muscle damage since larvae with substantial cellular damage swim poorly or erratically. The ability to swim properly should be determined by comparison to appropriate controls. A concentration-response curve can be generated and an EC50 (locomotion) determined.

X2.1.3 Hatchability- The embryos hatch from the fertilization membrane between 18 and 30 hrs. The number failing to hatch at 48 hrs should be recorded. Delay or failure indicates a slowing of developmental processes. This is analogous to staging the embryos at the end of the 96-hr time

Table 1. Ecological and Reproductive Characteristics of Amphibian Test Species* (23)

| Family and Species | Reproductive Characteristics | | | | Geographic Distribution | | Production | Remarks |
|--------------------|------------------------------|----------------------|--------------------|--------------------|--|------------------------------|------------|-----------|
| | Days of Incubation | Hatching Phase | Days of Incubation | Days of Incubation | Family (North America) | Species (North America) | | |
| Anaxyrus | 4 (11°C) | 308-360 ¹ | 100-150 hrd | 100-150 hrd | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |
| Bombina | 3 (11°C) | 48-60 ¹ | 4000-10,000 | 4000-10,000 | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |
| Hyla | 3 (11°C) | 41-41 ¹ | 30-40 hrd | 30-40 hrd | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |
| Rana | 3 (11°C) | 20-30 ¹ | 10-15 hrd | 10-15 hrd | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |
| Microhylis | 3 (11°C) | 20-30 ¹ | 10-15 hrd | 10-15 hrd | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |
| Bombina | 4 (11°C) | 40-60 ¹ | 3000-4000 hrd | 3000-4000 hrd | Cold to temperate regions of North America | Western US except Pacific NW | Temperate | Scattered |

* Data for reproduction characteristics and geographic distribution were taken from (1) Bishop (1942); (2) Bishop (1942); (3) Bishop (1942); (4) Bishop (1942); (5) Bishop (1942); (6) Bishop (1942); (7) Bishop (1942); (8) Bishop (1942); (9) Bishop (1942); (10) Bishop (1942); (11) Bishop (1942); (12) Bishop (1942); (13) Bishop (1942); (14) Bishop (1942); (15) Bishop (1942); (16) Bishop (1942); (17) Bishop (1942); (18) Bishop (1942); (19) Bishop (1942); (20) Bishop (1942); (21) Bishop (1942); (22) Bishop (1942); (23) Bishop (1942).

period except that it is much easier to score hatching. A concentration-response curve can be generated and a EC50 (hatching) determined.

X2.2 Additional Exposures

X2.2.1 Additional Exposure Length- In special circumstances, exposure periods exceeding 96 hr and/or pulse exposures may be performed. Data so collected should be reported as deviating from standard FETAX.

X2.2.2 Static- In the static technique, the test material is added at the beginning of the test and not changed. It should be recognized that many test materials will undergo loss in a short period of time. The static technique should only be used for materials that are extremely stable and do not volatilize or sorb to the test dishes. The cost or the size of the sample might also dictate that the static technique be used. This variation in procedure must be reported as deviating from standard FETAX.

X2.2.3 Flow-through- A toxicant-delivery system is used to continuously deliver toxicant and dilution water to the embryos. Small glass containers with bottom screening are used to contain the embryos in a larger diluter apparatus. The flow-through technique is recommended for chemicals that degrade quickly or are volatile or for large volume environmental samples. Every attempt should be made to use FETAX solution as the diluent. This variation in procedure must be reported as deviating from standard FETAX.

Atlas of Abnormalities



A Guide for the Performance of FETAX

by:

John A. Bantle, Ph.D.

James N. Dumont, Ph.D.

Robert A. Finch, Ph.D., D.A.B.T.

Gregory Linder, Ph.D.

John A. Bantle, Ph.D.

Department of Zoology
430 Life Sciences West
Oklahoma State University
Stillwater, OK 74078

James N. Dumont, Ph.D.

Oak Ridge National Laboratory
Bldg. 4500 North, MS 6186, RM 147
Oak Ridge, TN 37831-6186

Robert A. Finch, Ph.D., D.A.B.T.

U.S. Army Biomedical Research and Development Laboratory
Ft. Detrick, Bldg. 568
Frederick, MD 21702-5010

Gregory Linder, Ph.D.

N.S.I. Technology Services
200 S.W. 35th. Street
Corvallis Environmental Research Laboratory
Corvallis, OR 97333

Preface

The increasing costs of performing mammalian-based developmental toxicity tests, as well as the desire to reduce the number of laboratory mammals utilized in these tests, has stimulated the development and validation of short-term non-mammalian screening tests to assess the developmental toxicity potential of a wide variety of pure-chemicals and complex mixtures. We believe that the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) will provide a rapid, low cost alternative to the mammalian tests currently in use. FETAX was first developed by Dr. James Dumont and his co-workers at Oak Ridge National Laboratory in 1983. The assay originally was developed to assess the potential developmental toxicity of complex mixtures derived from the synthetic fuels program. The assay is based on a large body of information generated by a number of researchers in studies on normal embryonic development in which the developing *Xenopus* embryo was used as the model system.

The assay, as it is currently performed, is useful in screening for the potential developmental toxicity of both single chemicals, such as pharmaceuticals or commodity chemicals, and complex chemical mixtures, such as environmental samples. Research is in progress to identify suitable carrier solvents for non-water-soluble test samples, to develop an exogenous metabolizing system utilizing liver microsomes to allow preteratogens to be tested in the assay, and to develop methods so the assay can be utilized under field conditions for testing environmental samples.

This atlas is intended as a companion to the American Society for Testing and Materials (ASTM) standard guide for conducting the FETAX assay. The information and illustrations contained in this atlas provide a basis for the initial establishment of the assay in a laboratory, as well as a guide in the identification and interpretation of developmental abnormalities observed in embryos during the performance of the assay.

Preparation of this atlas has been supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-88-C-8031. The views, opinions, and/or findings contained in this document are those of the authors and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

Research was conducted in compliance with the Animal Welfare Act, and other Federal Statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication 86-23, 1985 edition. Citations of commercial organizations or trade names in this atlas do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

John A. Bantle, Ph.D.
James N. Dumont, Ph.D.
Robert A. Finch, Ph.D., D.A.B.T.
Gregory Linder, Ph.D.

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Chapter 1

Adults and Adult Care

This chapter covers sex determination, human chorionic gonadotropin (HCG) injection, and amplexus. Only a limited discussion of diseases will be provided because the frogs are normally quite hardy. Additional details of adult care are contained in the ASTM standard guide for FETAX.

FETAX uses *Xenopus laevis* embryos; other species of *Xenopus* should not be used. Healthy animals can provide embryos for several years. Females are

significantly larger than the males and those with a nose to rump length of 9 to 14 cm should be used. Males should be 6 cm or greater in length. Males and females should be rested 60 to 90 days between matings. Fewer eggs are produced if matings occur at less than 60 day intervals and a higher percentage of bad eggs are produced if intervals between matings extend beyond a 90-day period.

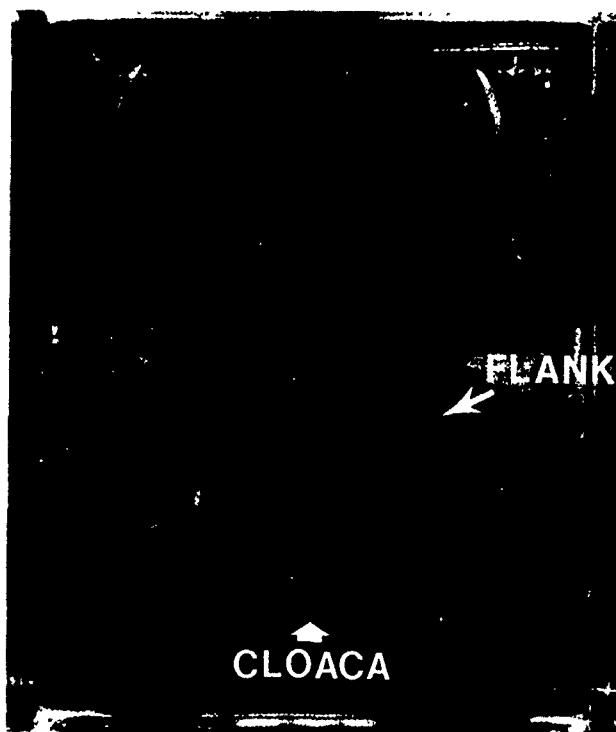


Figure 1-1. Normal Adult Female.

Although females are larger than males, size should not be used to determine sex. Females are easily identified by their fleshy cloacal lips which increase in size following HCG injection. Gravid females that have not been recently bred often show bulging flanks as the ovaries expand. This particular animal is not ready for breeding (see Figure 1-9 for comparison). Coloration will also vary considerably since skin color changes with surroundings (photo by Hull).

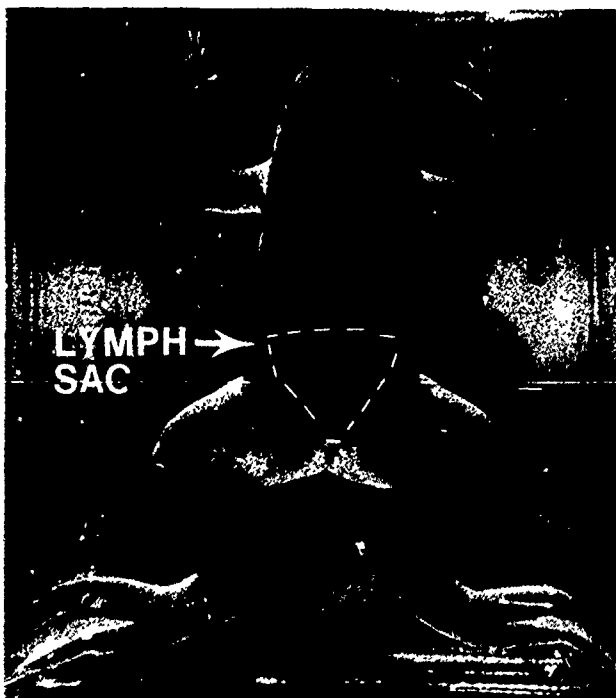


Figure 1-2. Normal Adult Male.

Males lack fleshy cloacal lips but possess dark forearm pads (nuptial pads) on the ventral surface of the hand and forearm which darken even further after HCG injection (see Figure 1-7). The pale marks on the back of this male are the result of liquid nitrogen branding for identification purposes. It is not necessary to brand frogs unless a large colony is to be maintained. With branding, frogs can be kept in community tanks while individual data are maintained regarding breeding success and date of mating. To brand a frog, place the animal in water containing 1.5 g/L of MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma Catalog # A 5040) until anesthetized. Remove the frog and place it on a wet paper towel. Dip a 1/8" diameter wire into liquid nitrogen for 20 seconds, apply it to the back skin for 6-8 seconds, and wait 24 hours for the brand to appear. Sixty days should elapse before mating is attempted in order to obtain complete recovery. Branding marks last at least one year. One alternative to branding is toe clipping. Various schemes can be devised to identify toe clipped animals (See: Roberts Rugh "Experimental Embryology-Techniques and Procedures," Burgess Publishing Co, Minneapolis, page 462, 1968). However, clipped toes regenerate and the procedure must be repeated every six months. Identification schemes employing color coded beads affixed to the skin via stainless steel wire have been devised but we have experienced trouble with wire loops ripping loose. Although initially traumatic, branding seems to be the current method of choice (photo by Hull).

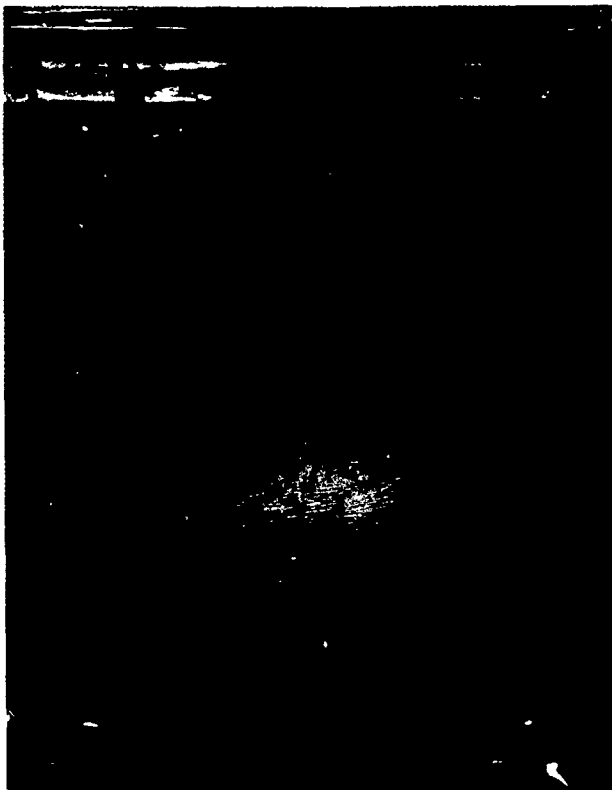


Figure 1-3. *Aeromonas* Infection.

Aeromonas hydrophila or "red-leg" is a bacterial infection that causes red blotches on both the abdomen and the ventral surfaces of the legs. Infected frogs must be immediately separated from the rest of the colony and intubated with Tetracycline (Nace, G., "Amphibians: Guidelines for the Breeding, Care and Maintenance of Laboratory Animals", National Academy of Sciences, ISBN 0-309-02210-X, 1974). Dissolve Tetracycline-HCl in tap water to a concentration of 25 mg/ml so that the intubation volume can be kept at about 0.2 ml. Dose the frog twice a day with 5 mg Tetracycline-HCl per 30 g body weight for one week. A small ball-tipped polyethylene stomach tube connected to a one cubic centimeter tuberculin syringe is used to administer the antibiotic. Severely infected animals, such as the one shown in Figure 1-3, should be destroyed (photo by Hull).



Figure 1-4. Nematode Infection in Adults.

By far the most common infection is that caused by nematodes. At the onset of infection the skin is rough to the touch and the frog does not feed actively. In more advanced stages, the skin turns white and lesions form. The frog shown in Figure 1-4 has begun to develop lesions on the back. Finally, the skin will slough off and the frog will waste away. To confirm nematode infection scrape a lesion with a single edged razor blade, deposit the scraping on a microscope slide and add a drop of water and a coverslip. Observe under 100X using a compound microscope (see Figure 1-5)(photo by Hull).



Figure 1-5. Nematode Present Among Skin Epithelial Cells.

Nematodes (arrow) are easily seen among the masses of epithelial cells. Once a member of the colony becomes infected, nematodes are likely to be found on all frogs in the colony. The best preventive measure is to treat the frogs as they arrive and to keep the colony as free as possible from infestation. For treatment, prepare a 4 gm/30 ml tap water stock solution of Thiabendazole (TBZ) which can be purchased from most agricultural stores and Sigma (Catalog # T 8904). TBZ is a treatment for nematodes in cattle. Add 1.5 ml TBZ solution to 2 liters of adult rearing water (dechlorinated or well water). Pour this solution into a large beaker containing two to three frogs. Treat overnight and repeat the treatment two weeks later. Do not mix treated and untreated frogs (photo by Fort).



Figure 1-6. Trematode with a Stage 11 *Xenopus* embryo.

Monogenetic trematodes can infect the bladders of adult frogs. During egg laying, they are deposited with the eggs and frequently attach themselves to the egg surface. The infected frog often shows dark blotches on the skin. The trematode on the left is shown next to a typical 1.5 mm diameter gastrula. In order to treat adult frogs for trematode infection, place the frogs in a large bucket containing 5 ml/10 gallon dechlorinated water of Mintezol (Merck, Sharp and Dohme) for 5 hours. Repeat this treatment in two weeks (photo by DeYoung).

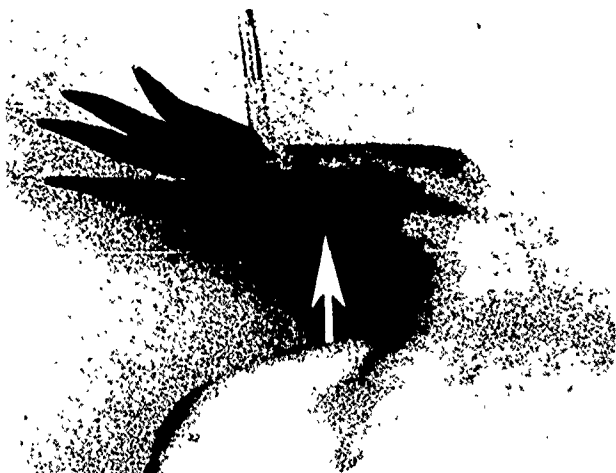


Figure 1-7. Male Forearm Pad.

This is a typical male forearm pad 24 hours after injection with 400 I.U. HCG. The pads assist in clasping the female during amplexus (see Figure 1-9) (photo by Hull).

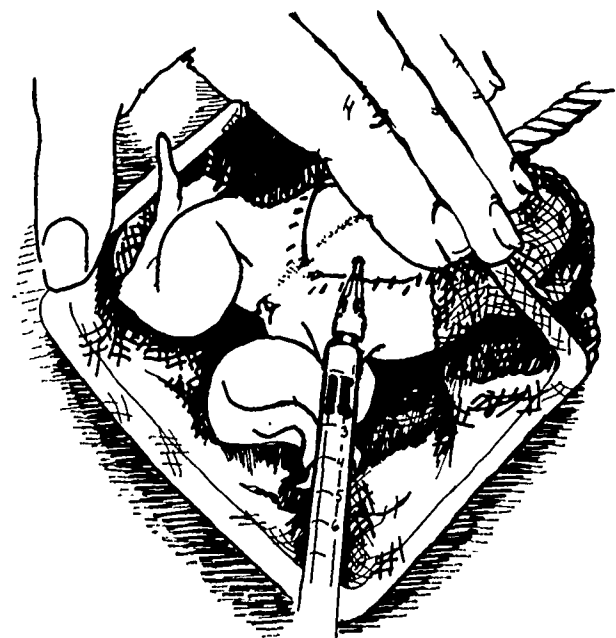


Figure 1-8. Method of Injecting Human Chorionic Gonadotropin (HCG).

It is easy to inject *Xenopus* if the animals are carefully immobilized. This is accomplished by lightly restraining the adult in an ordinary aquarium net. Use a tuberculin syringe with a 1/2" long, 26 gauge needle to inject the HCG into the dorsal lymph sac. The lymph sac is bounded by the lateral line which runs along the side of the frog and appears as "stitching" on the skin. The dorsal lymph sac is surrounded by a dashed white line in Figure 1-2. Injections should be inside (centrad) the lateral line. Note that one fourth of the needle tip enters the skin at a shallow (10-15 degree) angle. When injecting the frog, wrinkle the skin so that the injection can be administered subcutaneously. Be sure the needle tip penetrates into the lymph sac and not just into the skin. If only the skin is penetrated, a blister of fluid usually appears as the injection takes place. However, the lymph sac fills with a triangular shape if the injection is done properly. Keep the point of the needle well away from the spinal cord (drawing by DeYoung).



Figure 1-9. Amplexus in the Mating Tank.

A male and female frog are shown here in amplexus. A ten gallon glass aquarium has been used as a mating chamber. A plastic screen made from a fluorescent light diffuser grate (available from most hardware stores) has been used as a grate to support the adults. Mating is carried out in the dark at a temperature of 23 °C. FETAX solution (ASTM Standard Guide) is used as the medium and there should be 2.5 inches of solution above the grate in the mating chamber. The eggs fall to the bottom where they can be scraped into plastic Petri dishes. Another useful grate material is a 1 cm plastic mesh (Catalog # XV 0350) manufactured by InterNet Inc. [2730 Nevada Ave. North, Minneapolis, MN 55427; phone (612) 541-9690]. However, this must be purchased in large quantities. Heavy duty aluminum foil is used as a top and a bubbler is used for aeration. If a tank with shorter sides is used, a weighted lid may be necessary to prevent escape (photo by Hull).

Chapter 2

Staging and Egg Selection

The normal tables of *Xenopus* development by Nieuwkoop and Faber (Nieuwkoop, A.P. and Faber, J., Normal Tables of *Xenopus laevis* (Daudin). 2nd Ed., North Holland, Amsterdam, 1975.) are used in staging embryos for FETAX. However, they do not cover abnormal development and it is important to choose only normally developing embryos. The use of abnormal or over-ripe eggs would lead to unacceptably high mortality and malformation in controls. The Nieuwkoop and Faber (1975) staging guide is now out of print so this chapter contains enough normal staging information so that FETAX can be properly conducted. In order to select only normally cleaving embryos, a double selection process is used. Cleaving embryos should be scooped up in a small polystyrene Petri dish and placed in a 125 ml Erlenmeyer flask. The embryos are dejellied in 2% w/v cysteine-

FETAX solution for 2-3 minutes with swirling (see Figure 2-2). After the jelly is removed, the embryos are placed in large polystyrene Petri dishes. Do not keep them at high concentrations of cysteine or the degradation of overripe unfertilized eggs will affect the survival of otherwise normal embryos. In sorting the embryos, quickly remove poorly pigmented and enlarged eggs first. Then, carefully select only normally cleaving embryos and place them into separate dishes containing FETAX solution. Transfer the eggs with a glass Pasteur pipet that has had the tip broken off and the end fire-polished. Repeat the selection process a second time and choose only the best embryos. Control mortality and malformation can be kept to less than 5% when proficiency in selection has been developed.



Figure 2-1. A Typical Blastula.

This figure shows a dorsal view of a typical *Xenopus* Stage 8 blastula as it might be collected from the bottom of the breeding aquarium. The jelly coat has not been removed. It is quite sticky and attempts to manipulate the embryo with a spatula will prove time consuming (photo by Hull).

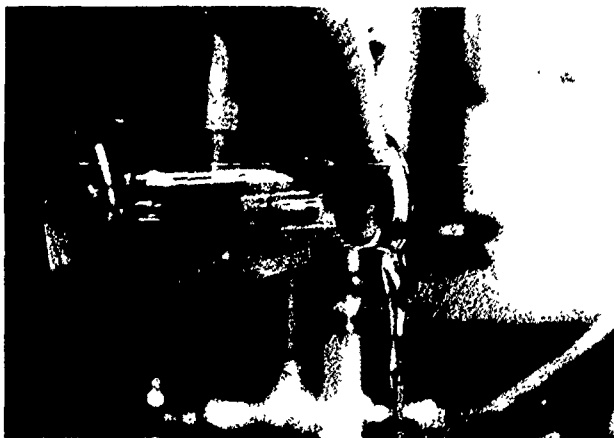


Figure 2-2. Decanting the 2% Cysteine after the Dejelling Process.

The embryos are dejellied as described in the ASTM Standard Guide. Great care must be taken not to over-treat the embryos or they will disintegrate. Note that the water becomes cloudy as the jelly is removed. As the embryos begin to roll freely in the flask, stop the gentle swirling motion and let them settle to the bottom of the flask. Pour off the cysteine solution as shown. Add FETAX solution, swirl briefly and decant. Repeat two times (photo by Hull).



Figure 2-3. Normal Clutch of Unfertilized *Xenopus* Eggs.

Normal unfertilized eggs have a uniformly pigmented animal hemisphere and a white to cream-colored vegetal hemisphere. Several layers of jelly surround each egg and the eggs can form a large mass. The eggs can be scraped off the bottom of the breeding aquarium by using a plastic Petri dish. Place the dish upside down on the bottom of the aquarium and slide it along the bottom. The eggs will form a mass along one edge. Gently invert the dish so that you raise the edge with the eggs first. With a little practice, the eggs will flip into the dish as you turn it over (photo by DeYoung).



Figure 2-4. Eggs Laid in a String.

This is an example of eggs laid in a string. Some of the eggs may appear normal but most are poorly pigmented and are not round. Even with careful selection many develop abnormally. This type of egg and embryo should not be used in FETAX (photo by Bantle).

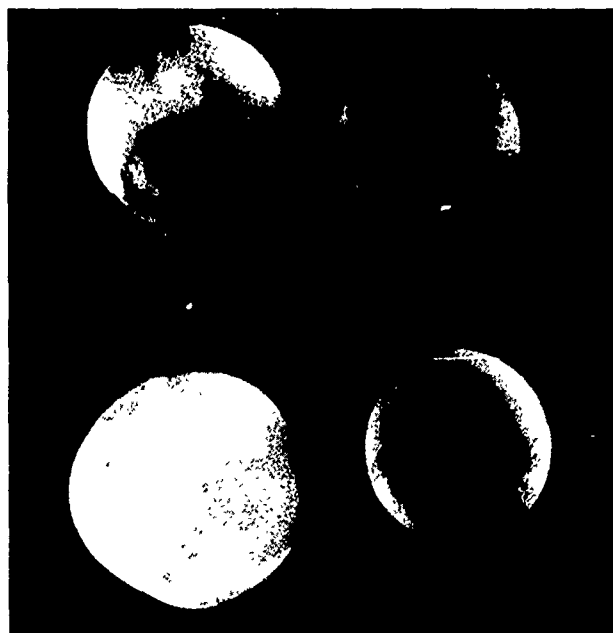


Figure 2-5. Necrotic Eggs.

These eggs are necrotic as indicated by the lack of uniformity in the pigmentation pattern. They should be removed as soon as possible during the selection process (photo by Hull).

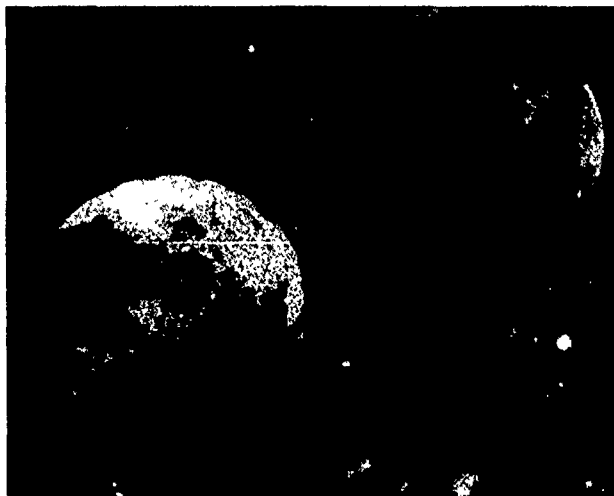


Figure 2-6. Embryos Not Perfectly Round.

Only perfectly round blastulae should be selected for FETAX. The pigmentation of these is irregular. Although the jelly coat has been removed from these blastulae, the fertilization membrane is still intact although it is difficult to observe in this photograph (photo by DeYoung).



Figure 2-7. Yolk Leaking from Blastulae.

Yolk is leaking from these blastulae (arrow) and occupying the space between the fertilization membrane and the oolemma. Any embryo showing this type of leakage should be rejected during the selection process (photo by Hull).

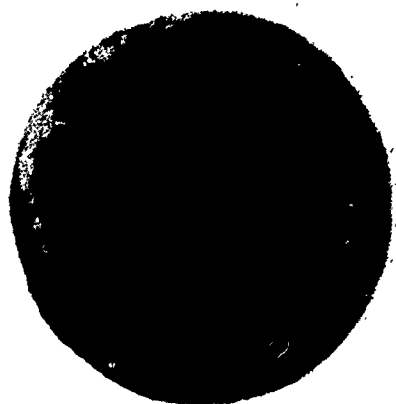


Figure 2-8. Blastula with Abnormal Pigmentation.

This large cell blastula has abnormal pigmentation. The pigment is poorly distributed; it should be localized in the animal hemisphere. This type of pigmentation indicates that the embryo will not develop properly and, therefore, it should be rejected for use in FETAX (photo by Hull).

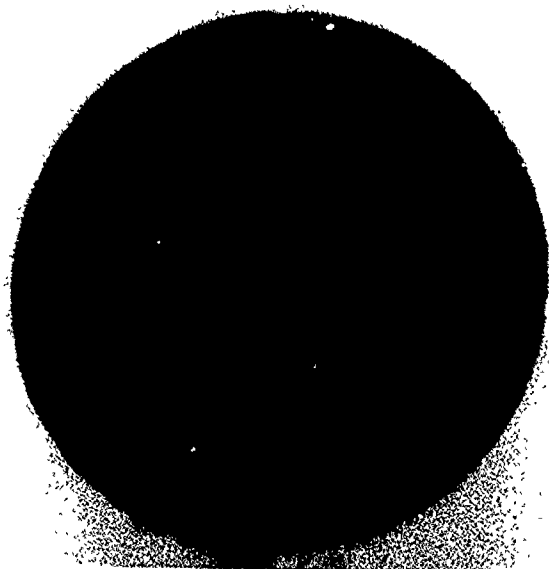


Plate A

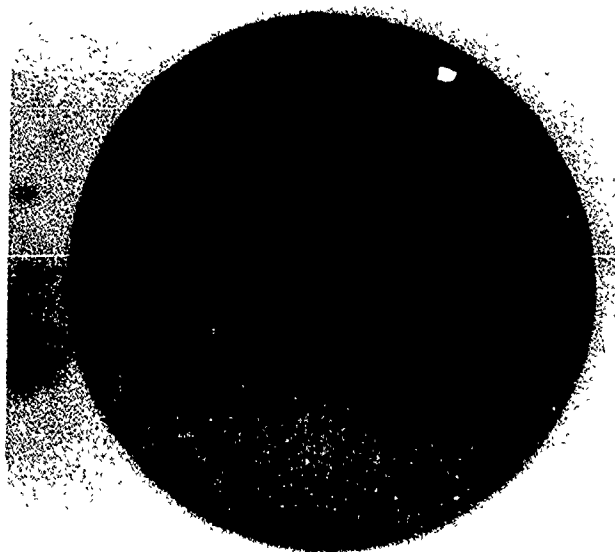


Plate B

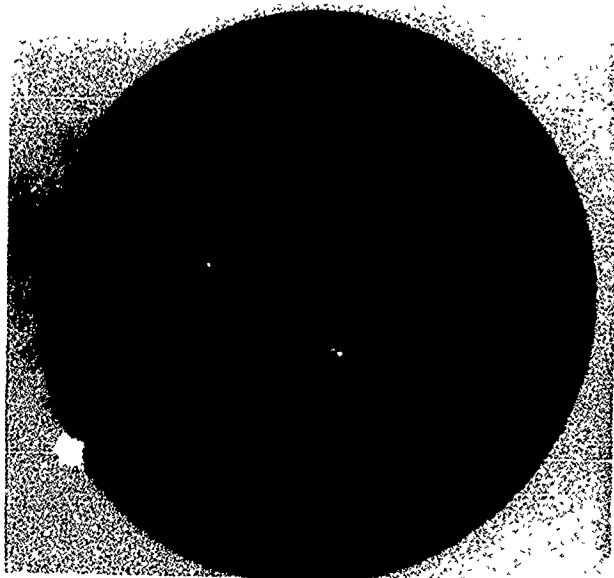


Plate D

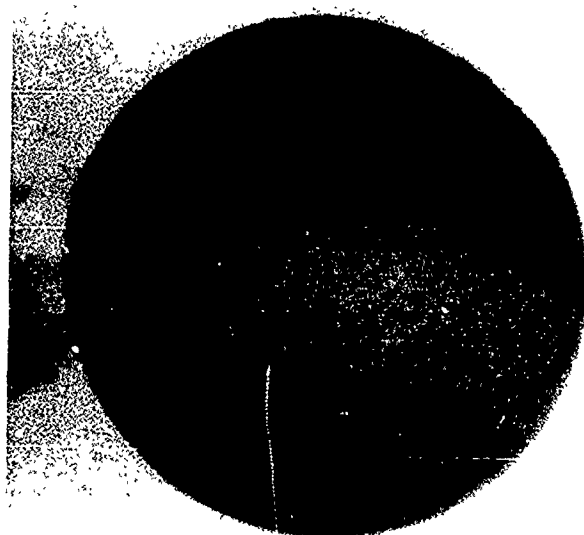


Plate E

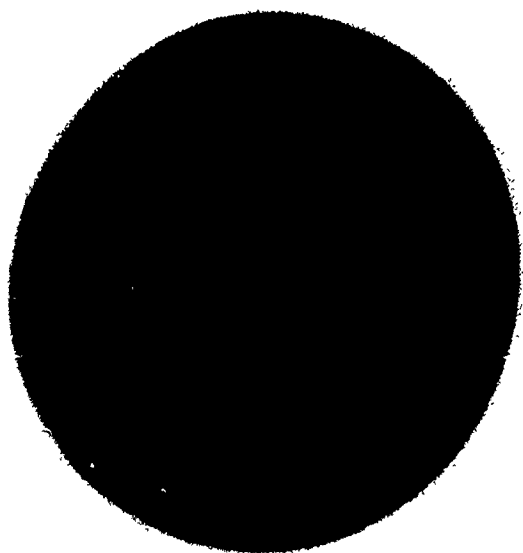


Plate G

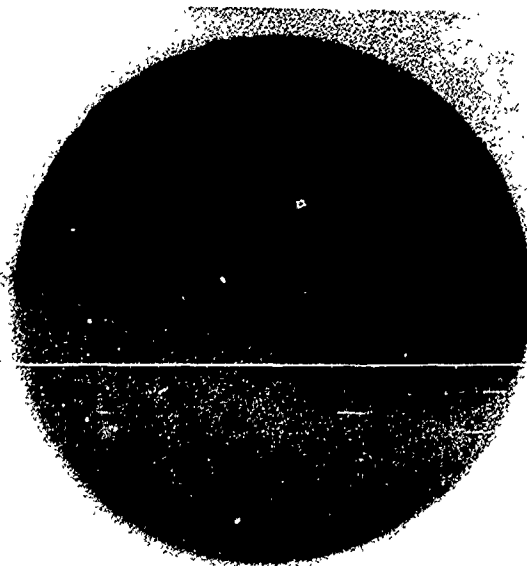


Plate H

Figure 2-9. Normal Stage 7.5 Blastulae.

Plate A shows a dorsal view of the earliest stage of development that can be used for FETAX (the ASTM guide recommends the use of Stage 8). By this stage, normal cleavage and development can be ascertained. Note that the pigmentation of this embryo is slightly irregular. It did not affect development of the embryos from this clutch. Plate B shows a lateral view of a similar blastula. The vegetal hemisphere cells are creamy white to white and always larger than the pigmented animal hemisphere cells. The pigmentation line is at the equator of the blastula. Diagram C illustrates the relative cell sizes of animal and vegetal hemisphere cells and the extent of migration of pigmented animal hemisphere cells down across the surface of the vegetal hemisphere (photo by Hull; diagram by DeYoung).

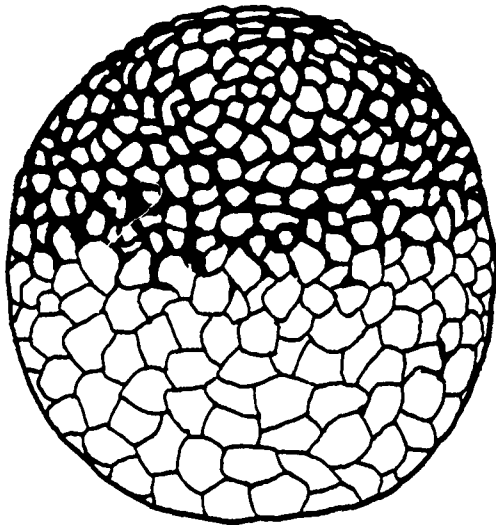


Diagram C

Figure 2-10. Normal Stage 8 Blastulae.

Plate D is a dorsal view of a medium cell blastula. Plate E is a lateral view that happens to be tilted away from the viewer. Notice that there is a gradual reduction in size of the cells in the animal hemisphere area compared to the size of the cells in the vegetal hemisphere. Diagram F is a lateral view blastula illustrating the gradual reduction in cell size and the progressive movement of the pigmented animal hemisphere cells down over the larger white vegetal hemisphere cells (photo by Hull; diagram by DeYoung).

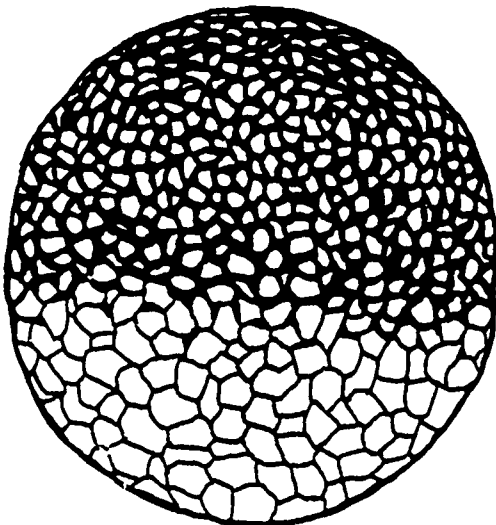


Diagram F

Figure 2-11. Normal Stage 8.5 Blastulae.

Plate G, a dorsal view of a fine cell blastula, shows that cell size has further been reduced. Generally, pigmentation is more even than that shown here. The lateral view in Plate H clearly shows that two-thirds of the blastula is covered with small pigmented cells. Cells at the equator are less pigmented than those at the top. Diagram I shows the movement of the small cells down over the vegetal hemisphere cells. In Plate H, the animal pole is tilted slightly away from the viewer (photo by Hull; diagram by DeYoung).

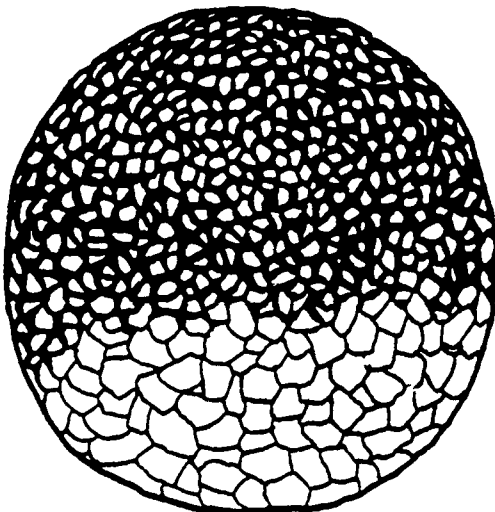


Diagram I

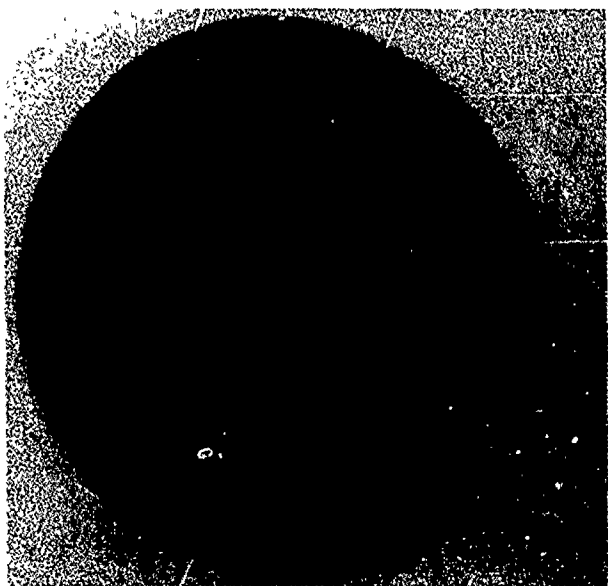


Plate J

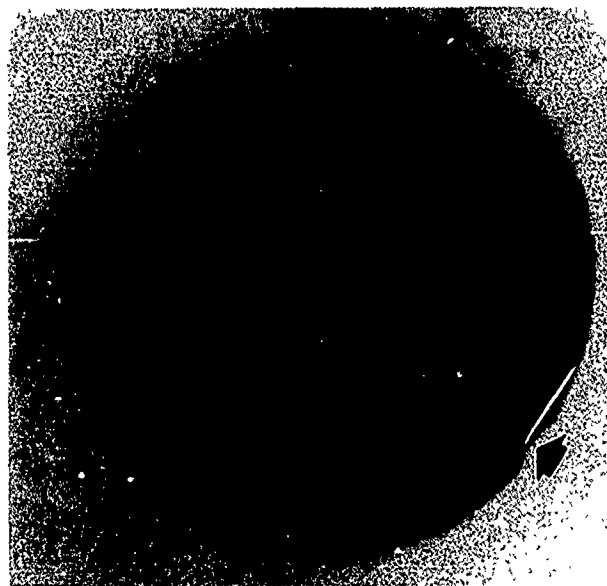


Plate K

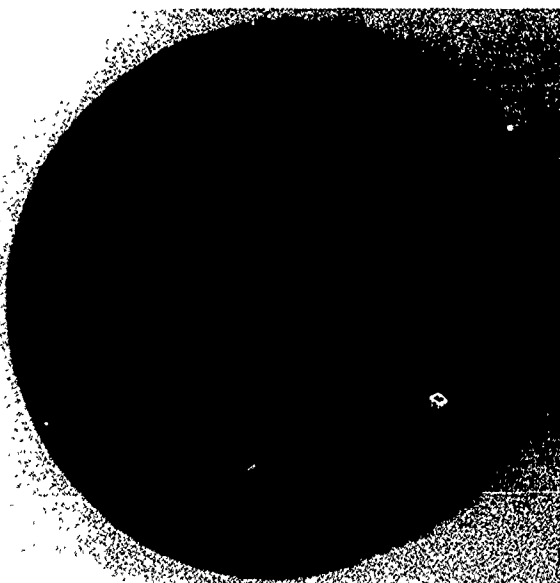


Plate M

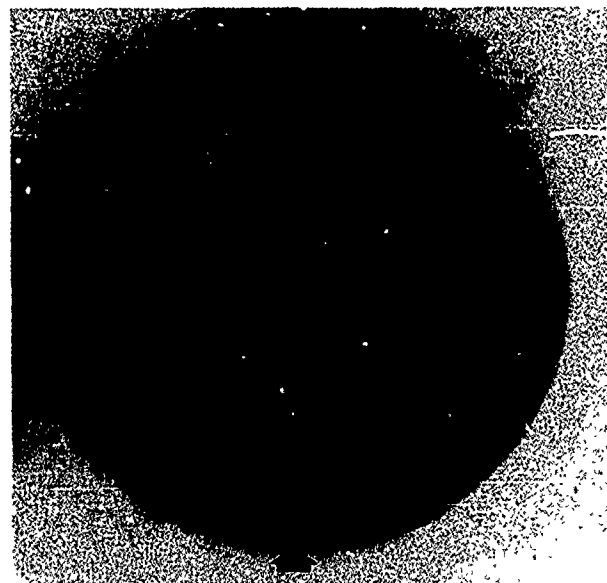


Plate N

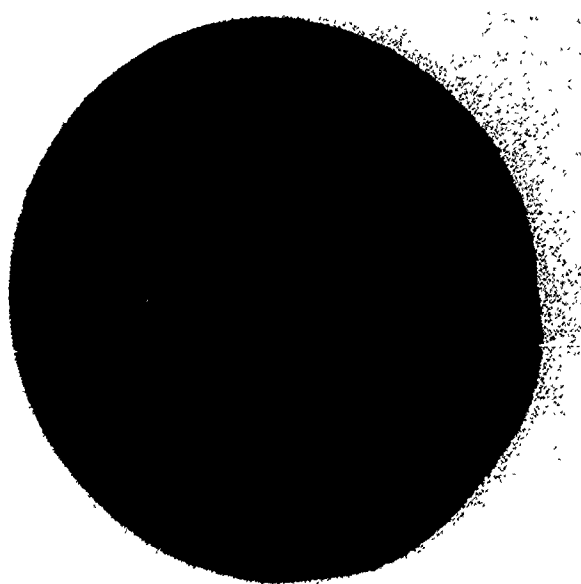


Plate P

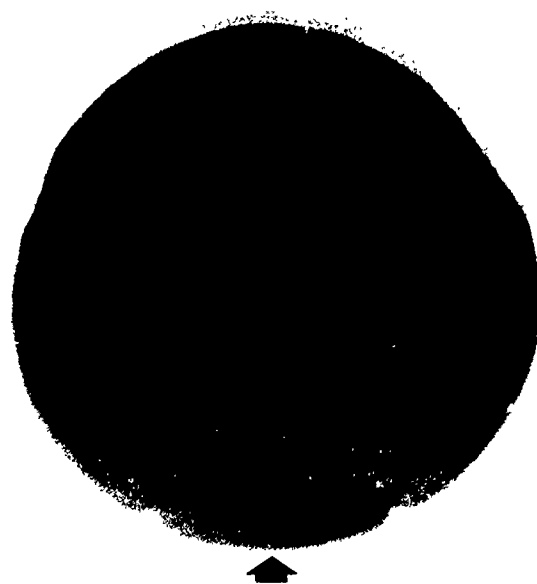


Plate Q

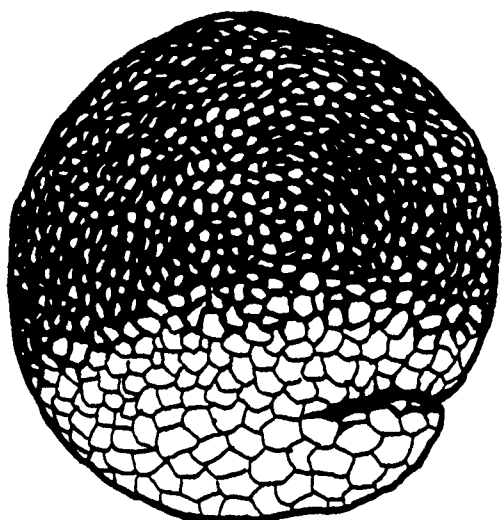


Diagram L

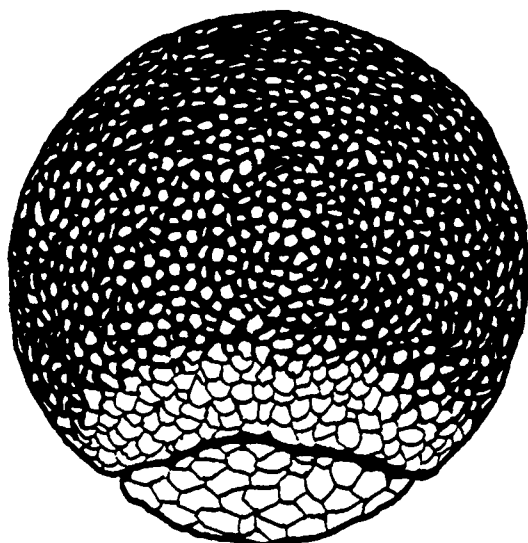


Diagram O

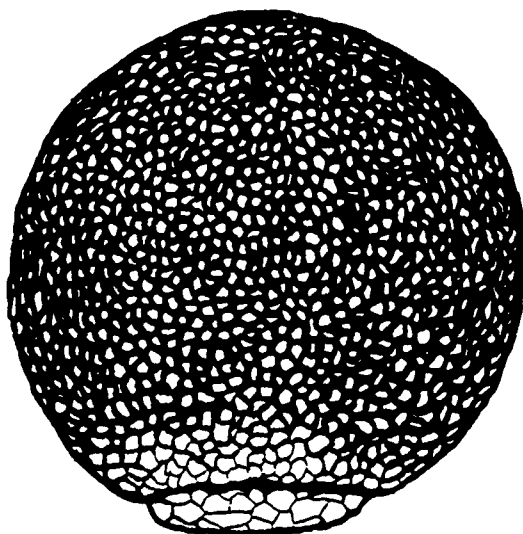


Diagram R

Figure 2-12. Normal Stage 10 Gastrulae.

Plate J shows a dorsal view of a Stage 10 gastrula. The embryo must usually be rotated on its side in order to see the dorsal lip of the blastopore. The arrow clearly shows the blastopore in Plate K. Diagram L shows the dorsal lip of the blastopore as a crescent-shaped crease below the equator of the embryo (photo by Hull; diagram by DeYoung).

Figure 2-13. Normal Stage 11 Gastrulae.

Plate M is a dorsal view while Plate N is the lateral view. The blastopore now encircles the lower part of the embryo and the white circle of yolk cells are now referred to as a yolk plug (arrow). Diagram O shows the extent to which the animal hemisphere cells have now enveloped the embryo (photo by Hull; diagram by DeYoung).

Figure 2-14. Normal Stage 11.5 Gastrulae.

As gastrulation proceeds, the size of the yolk plug decreases. This represents the last stage of development that can be used for FETAX. It is recommended that medium to fine cell blastulae be selected for use but, as sometimes happens, egg deposition takes place early and older embryos must be used. Plate P shows the dorsal view with extremely small cells dotting the surface while Plate Q shows the lateral view with reduced blastopore and yolk plug (arrow). Diagram R illustrates not only the migration of the animal hemisphere cells but the reduction of the size of the yolk plug (photo by Hull; diagram by DeYoung).



Figure 2-15. Ventral View of Stage 10 Gastrula.

Note the crescent-shaped blastopore on the vegetal hemisphere of the embryo. Compare to Figure 2-12 (photo by Hull).

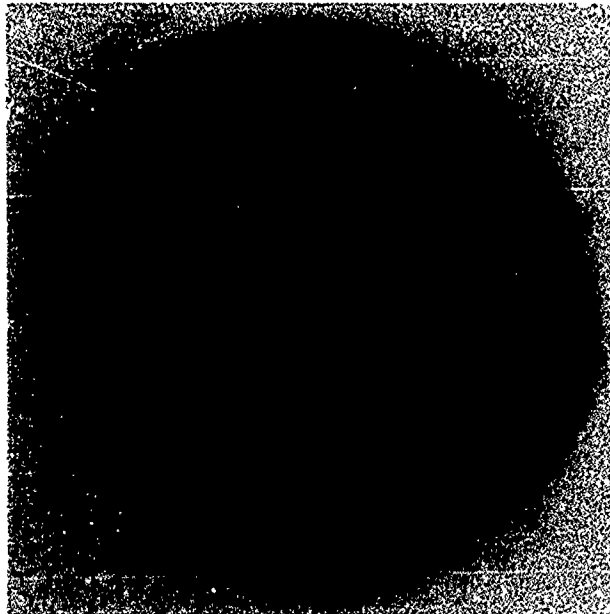


Figure 2-16. Ventral View of Stage 11 Gastrula.

The blastopore is now oval. Compare to Figure 2-13 (photo by Hull).

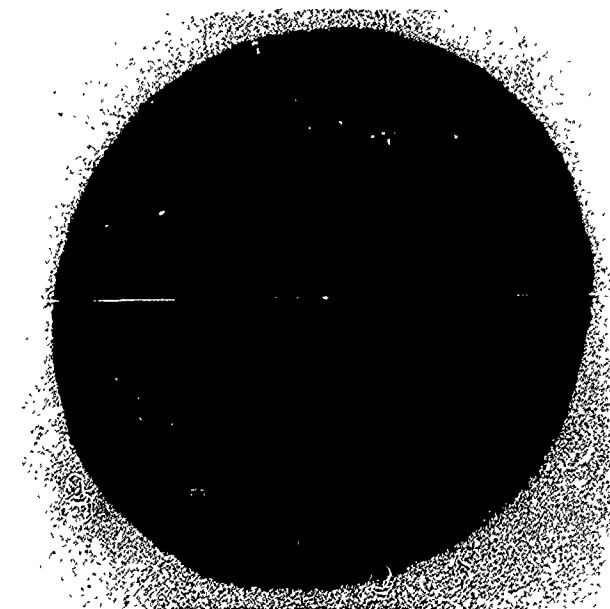


Figure 2-17. Ventral View of Stage 11.5 Gastrula.

The blastopore is more round and is reduced in size. Compare to Figure 2-14 (photo by Hull).

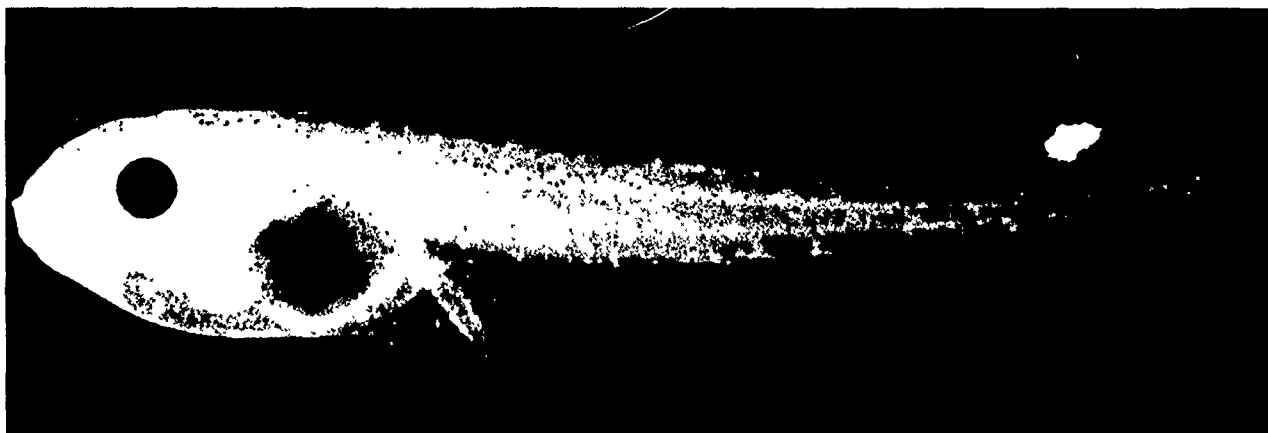


Figure 2-18. The Normal Stage 46 (96-hr) Larva.

A full length view. Refer to Figure 2-19 for structures (photo by Hull!).

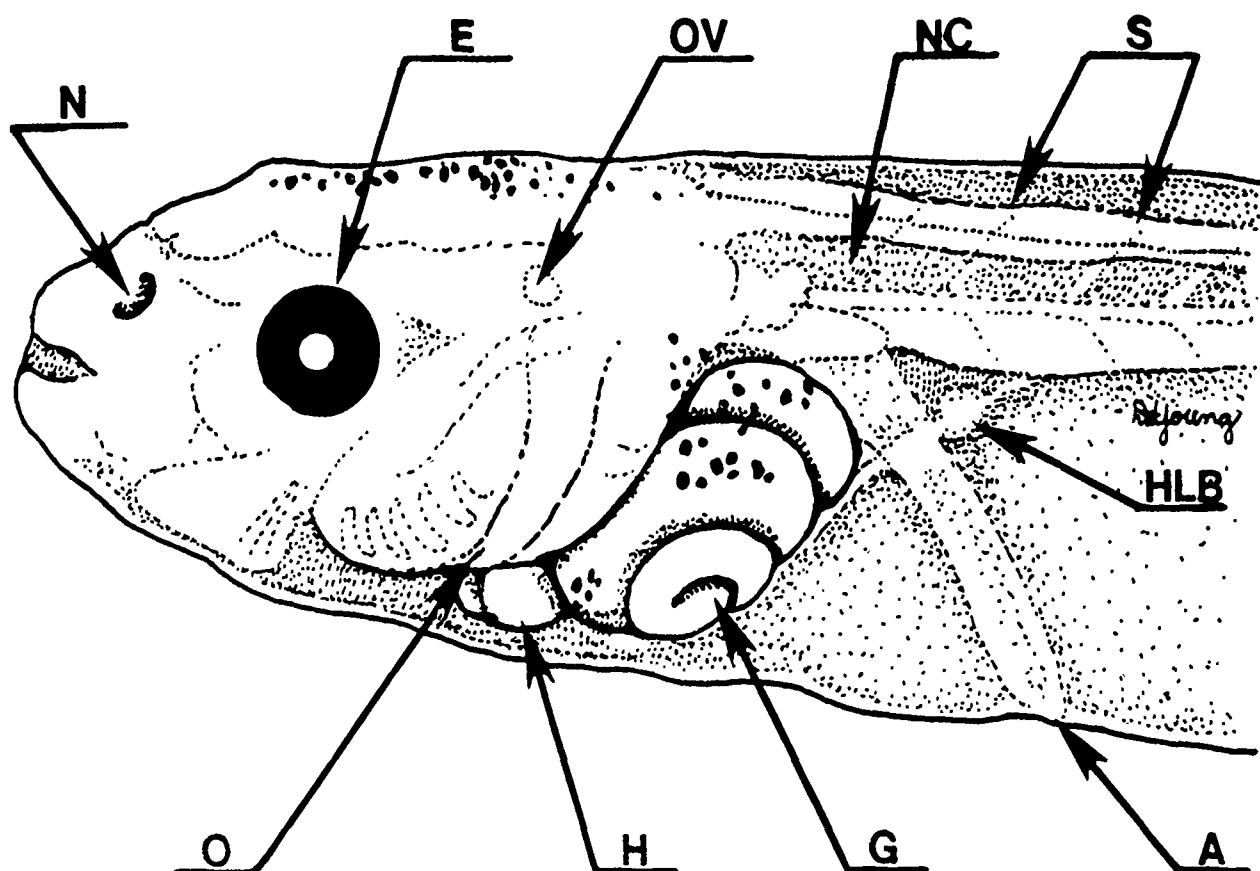


Figure 2-19. Diagram of the Head Region of the Stage 46 (96-hr) Larva.

A Stage 46 larva is recognized by the appearance of the hind limb bud, the coiling of the gut, and the shape of the operculum covering the gills. The best indicator that the larva has attained Stage 46 is the appearance of the hind limb bud. Gut coiling is also easily observed; N=nares, E=eye, OV=otic vesicle, S=somite, O=operculum, H=heart, G=gut, HLB=hind limb bud, A=anus (diagram by DeYoung).



Figure 2-20. Normal Stage 46 (96-hr) Larva.

It is important to expose the embryos in FETAX until the controls reach Stage 46. The three larva shown exhibit the gradual appearance of the hind limb bud. The bottom tadpole is only Stage 45 (about 4 days) and, therefore, does not exhibit the hind limb bud. The two embryos above it are Stage 46 (middle) and Stage 47 (top), respectively. FETAX should be completed at Stage 46 (photo by Hull).

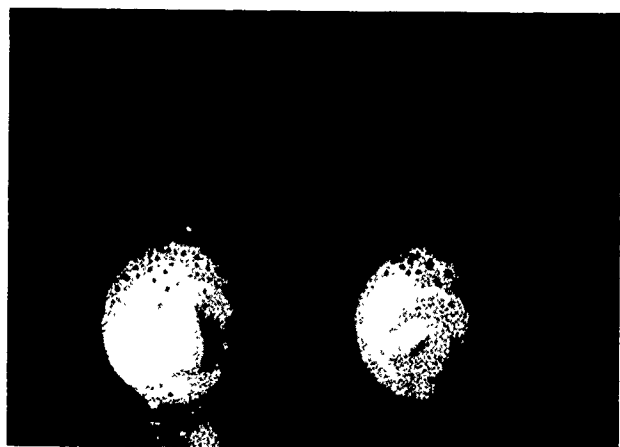


Figure 2-21. Normal Gut Coiling.

The tadpole on the left is Stage 45 and does not display complete tight gut coiling. The Stage 46 embryo on the right has developed a good tight complete coil (photo by Hull).

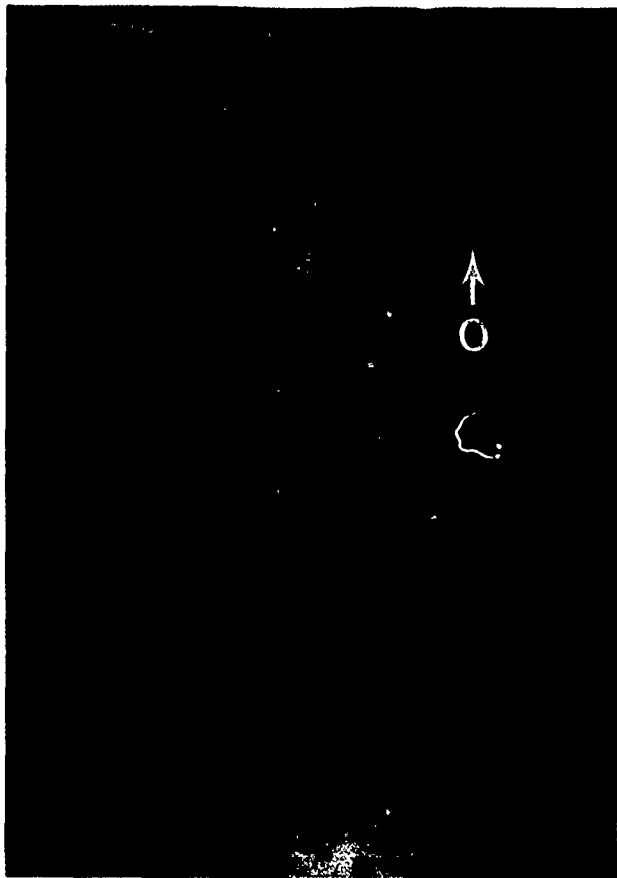


Figure 2-22. Dorsal View of the Normal Stage 46 (96-hr) Larva.

The lens of the eye is not visible. Note the size of the normal eye in relation to the rest of the body; O=optic stalk (photo by Hull).

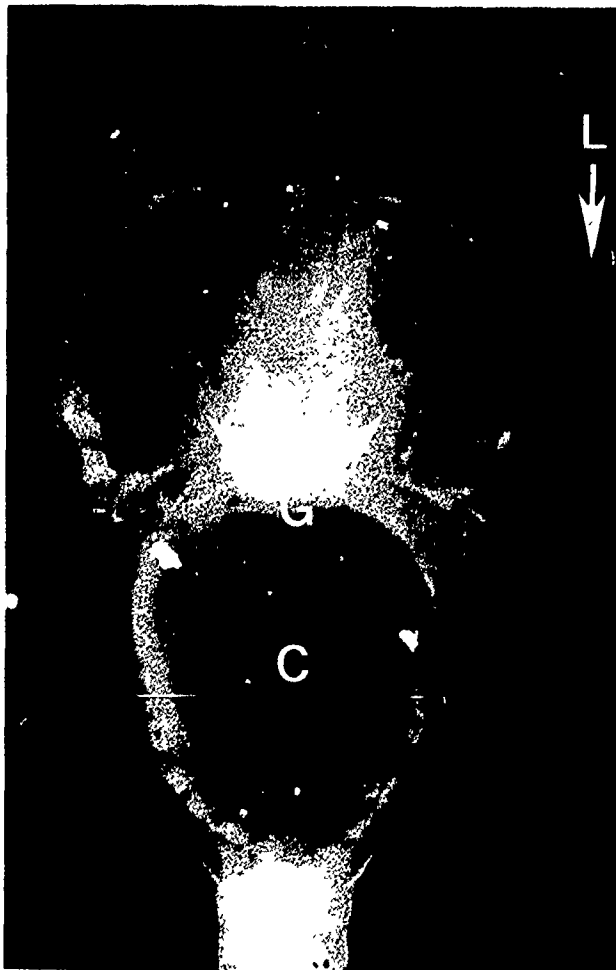


Figure 2-23. Ventral View of the Eye Region of a Normal Stage 46 (96-hr) Larva.

The lens of the eye is visible in this micrograph. Note the size of the eye and gut in relation to the rest of the body; L=lens, G=gills, C=properly coiled gut (photo by Hull).

Chapter 3

Axial Malformations

Because most abnormal embryos possess multiple malformations, it has been difficult to arrange the atlas photographs by type of abnormality. Therefore, the atlas has been arranged beginning with the most obvious (i.e. easily detectable abnormalities) through increasingly more complex examples. In each case, all abnormalities will be noted and the user is urged to consult previous or later photographs for more details. As one proceeds through the atlas, examples of specific malformations (terata) are shown in greater detail. Scanning electron micrographs and photographs of sectioned histological preparations are also presented throughout the atlas. Although these techniques are not needed in order to detect abnormalities

in FETAX, the data are none-the-less presented with the hope that they provide better descriptions and aid understanding of the abnormalities seen with the aid of the dissecting microscope.

Unless otherwise noted, in photographs where more than one embryo is shown, the embryos have been exposed to increasing concentrations of the same test material. Usually the top embryo represents either a control or an exposure at a low concentration. The remaining embryos will usually be arranged in order of increasing concentration. Appendix II contains information regarding test materials and the concentrations used.



Figure 3-1. Simple Minor Axial Abnormality.

A simple minor axial abnormality in which the tail is slightly dorsally curved, gives the impression of a slight arch (photo by Hull).



Figure 3-2. A More Complex Axial Abnormality.

Here is a more complex axial abnormality in which the distal portion of the tail is flexed ventrally and a slight dorsal flexure of the axis is present in the abdominal/cervical region (photo by DeYoung).



Figure 3-3. Lateral Flexure of the Tail.

This is a relatively mild lateral flexure of the tail. Such abnormalities are usually related to malformations of the notochord. This embryo also displays mild eye (ophthalmic) and abdominal edema; E=eye edema, A=abdominal edema (photo by DeYoung).



Figure 3-4. Severe Lateral Flexure of the Tail.

Tail abnormalities are frequently associated with the notochord as is the case with these embryos. In all other respects, the embryos appear normal (photo by Dumont).

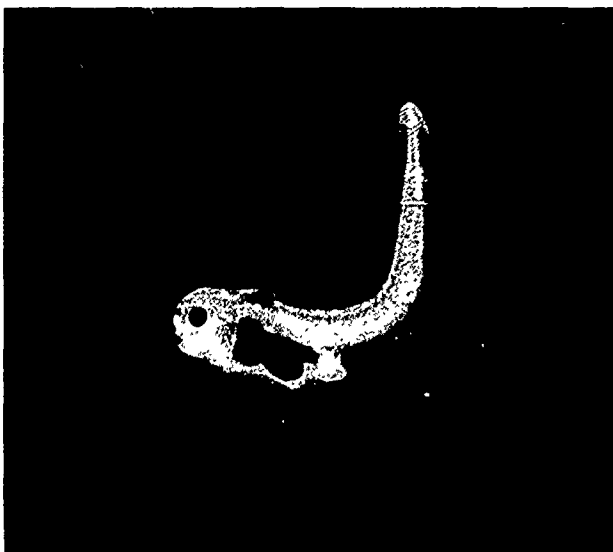


Figure 3-5. A Case of Dorsal Tail Flexure.

Pronounced dorsal flexure of the tail just posterior to the abdominal region is shown in this photograph. Reduced head development (microcephaly) and reduced coiling of the gut is also seen (photo by Dumont).



Figure 3-6. Curved Tail Malformation.

Severe axial abnormalities of the tail may include pronounced dorsal coiling of the tail as shown in these embryos. They also display reduced head development and significantly reduced coiling of the gut. Note that both embryos share the same defect. Some toxicants cause the same effects in all embryos (photo by Dumont).



Figure 3-7. Wavy Tail Malformation.

The embryo in the top of the photograph is a control while the one shown at the bottom illustrates a wavy condition of the tail with a slight dorsal flexure at the end. The notochord is clearly seen in this photograph and, along with somites, is abnormal. The abnormal embryo is shorter than normal and displays an incompletely coiled gut (photo by Dumont).



Figure 3-8. Severe Wavy Tail Malformation.

The embryo at the top is the control. The embryo in the middle shows an undulating or wavy notochord, while the embryo at the bottom has a stunted tail with few waves, severe abdominal edema, and abnormal gut coiling. There is also a progressive reduction in head size (photo by Hull).

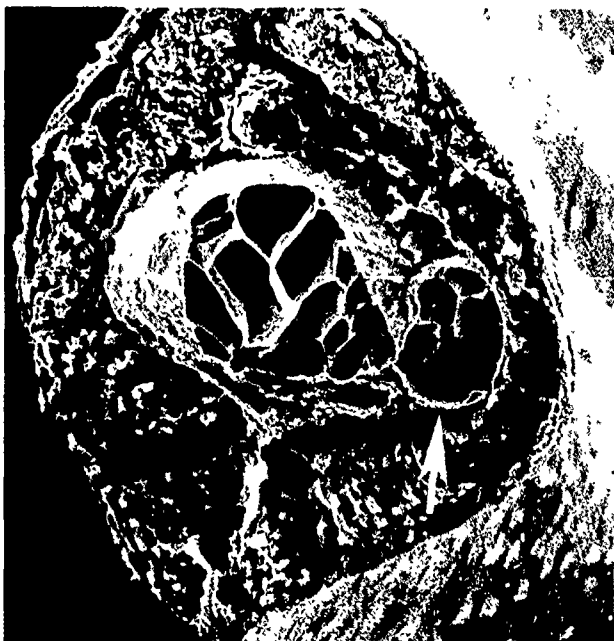


Figure 3-9. Cross-Section of a Stage 46 (96-hr) Embryo.

This scanning electron micrograph of a transected embryo tail shows a herniated region of the notochord (arrow). Herniations and curved, or wavy notochords are frequently seen in embryos that have been exposed to laythrogens (photo by Dumont).



Figure 3-10. Axial Shortening.

General shortening of the embryonic axis is frequently seen and may or may not be associated with other abnormalities. Histological studies have revealed that there is usually either a reduction in number or size of the somites. In this case, the embryo on the bottom is a control. The embryo in the top of the photo shows significant shortening (reduced growth). In such cases the tail fin frequently appears broad and may originate from a more anterior position (in this illustration near the middle of the dorsal abdominal region). The embryo also shows mild cardiac edema (arrow) and mild retardation of gut coiling (photo by Dumont).

Chapter 4

Blistering and Edema

Edema is a frequent occurrence and may be general (somatic) or regional, e.g., eye (optic), abdominal, cranial, or mallar. Edema is easily identified and appears as transparent, swollen, fluid-filled areas. Occasionally,

epidermal blisters may appear and these are also classified as edema. Their locations on the embryo varies. Mild edema is most easily identified in living embryos.



Figure 4-1. Embryos with Moderate Blistering.

Malformed embryos that display various degrees of blistering in the absence of severe edema are shown in this photo. Blisters (arrows) are most frequently observed along the dorsal midline (fin) area or ventrally near the anus. These embryos are also much shorter than normal, have malformed guts, and display reduced eye (microphthalmia) and head development (microcephalia) (photo by Dumont).



Figure 4-2. Optic Edema and Blistering.

This scanning electron micrograph of an embryo displays dorsal and ventral blisters. Severe optic edema (arrow), malformation of the head/face and reduced length are also apparent (photo by Dumont).



Figure 4-3. Cardiac Edema.

These embryos exhibit edema in the cardiac region (arrows) (control at top). Such edemas are frequently accompanied by malformations of the heart. The embryo at the bottom also displays malformations of the eyes, head, face, and gut (photo by Dumont).



Figure 4-4. Abdominal and Cardiac Edema.

This series of embryos display increasingly severe abdominal edema (arrows) after exposure to increasing concentrations of test material (control at top). Failure to achieve normal gut development often accompanies such edema. In this case there is an almost total failure of the gut to undergo normal torsion (coiling) (photo by Dumont).

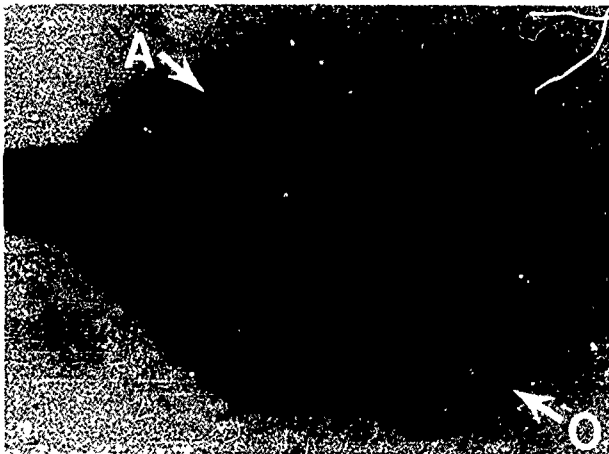


Figure 4-5. Mild Optic Edema.

This embryo has mild edema in the optic and abdominal areas; O=optic and A=abdominal (photo by Hull).

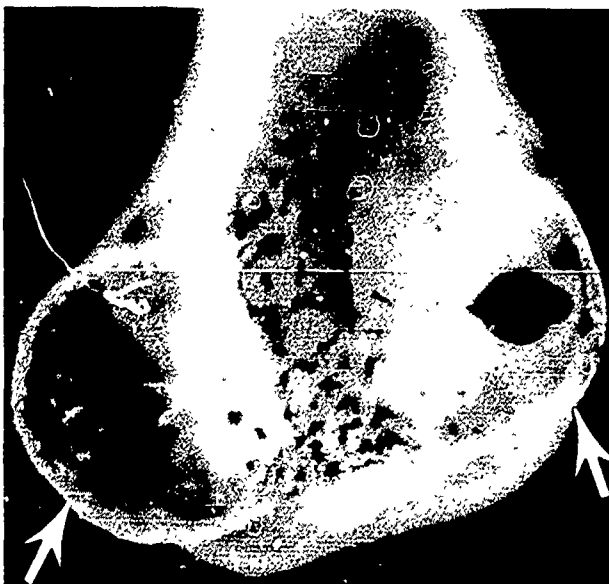


Figure 4-6. Severe Optic Edema.

An embryo has severe optic edema (arrows). Edematous areas are usually devoid of pigment. Optic edema is usually accompanied by malformations of the eye. In this case the eyes are reduced in size (microphthalmia) (photo by Hull).

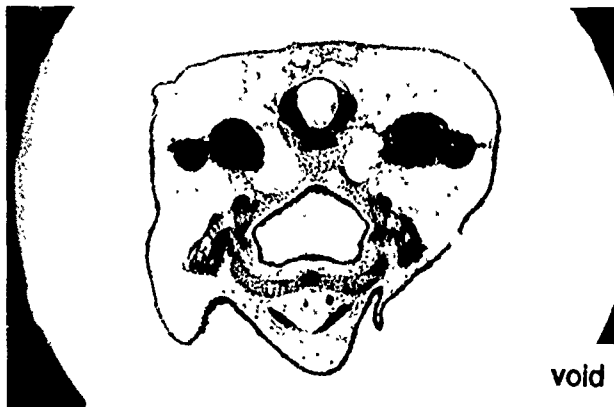


Figure 4-7. A Histological Section Through the Head Region of a Severely Edematous Embryo.

The mesenchymal tissue is dispersed and large "void" spaces are seen (arrow). In this case, the eyes are also abnormal, as the lenses appear to be developing outside the optic cup (photo by Dumont).

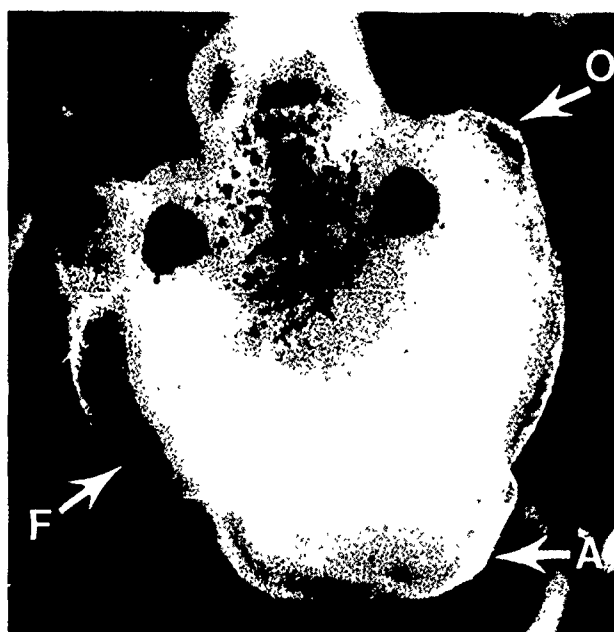


Figure 4-8. An Anterior View of a Severely Edematous Embryo.

This embryo has severe optic, facial and abdominal edema; O=optic, F=face, and A=abdominal (photo by Hull).



Figure 4-9. Severe Generalized Edema.

Severe abnormalities affecting all major organ systems, e.g., head, face, eye, brain, and gut are accompanied by extensive edema (photo by Dumont).

Chapter 5

Eye Abnormalities

Abnormalities of the eye include reduced development (microphthalmia), failure of the choroid fissure to close [closure is normally nearly complete at Stage 35/36 (2 days)], reduced or inappropriate pigmentation, dislocation of the lens, sometimes to an

area outside the developing optic cup, and failure of the optic cup to separate from the brain (diencephalon). Some of the more subtle anomalies may be difficult to detect. When detected in a single specimen, all embryos should be carefully examined.



Figure 5-1. Side View of the Eye Region of a Normal Stage 46 (96-hr) Embryo.

The eye is rather large, concentric and darkly pigmented. A pale, translucent lens is situated in the center, or slightly below the center, of the eye. The choroid fissure appears as a slit on the ventral aspect (arrow). It should be barely visible as an indentation in the ventral surface (photo by Hull).



Figure 5-2. Histological Cross Section of a Normal Stage 46 (96-hr) Embryo.

This is a cross section through the eye region at the same stage of development as in Figure 5-1; HE=head ectoderm (cornea), L=lens, I=iris, NR=neural retina, PR=pigmented retina, O=optic stalk (photo by Dumont).



Figure 5-3. Reduction in Eye Size.

Both eyes are smaller than normal in this mildly edematous embryo and the lens of the smaller eye appears to be extruding from the pigmented eye cup (arrow) (photo by Hull).



Figure 5-4. Reduction in the Size of a Single Eye.

It is important to carefully examine both eyes. Sometimes one is normal while the other may be abnormal as in this example where the left eye is greatly reduced in size. In all other respects, the embryo appears normal (photo by Hull).



Figure 5-5. Failure of the Choroid Fissure to Close.

Other eye abnormalities include incomplete closure (fusion) of the choroid fissure. This feature may range in severity from a slight enlargement of the gap to a very pronounced slit. In this embryo shown, closure of the fissure appears only partially incomplete (arrow) while the size of the eye and lens appear normal (photo by Hull).



Figure 5-6. Moderate Failure of the Choroid Fissure to Close.

Incomplete fusion of the choroid fissure is clearly seen in this embryo's eye (arrow). The eye is also reduced in size (photo by Hull).



Figure 5-7. Severe Failure of the Choroid Fissure to Close.

A complete failure of the choroid fissure to close (arrow) is evident in this embryo. The embryo also exhibits rather severe optic edema as well as facial abnormalities (photo by Hull).

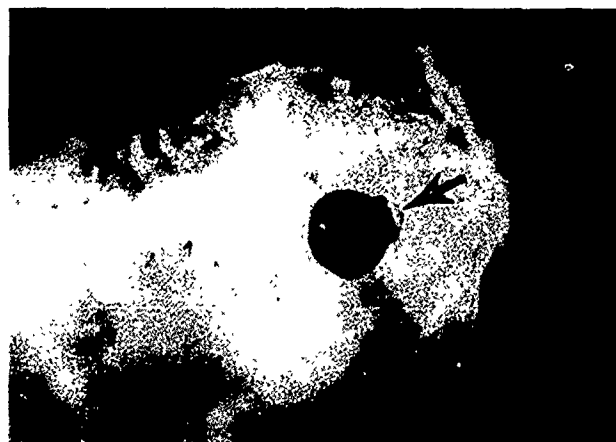


Figure 5-8. Optic Cup Rupture.

Ruptures of the optic cup sometimes occur in which it appears that the lenses are extruded (arrow). In other respects the eye appears normal. The embryo shown displays edema and facial malformations such as ocular edema and foreshortened facial features (photo by Hull).



Figure 5-9. Severe Optic Cup Rupture.

The eye of this embryo appears normal except for the accumulation of a mass of pigment on its dorsal surface (photo by Hull).



Figure 5-10. Progressive Depigmentation of the Eye.

Some developmental toxicants cause progressive loss of pigment. The control in this series (top) shows normal pigmentation while increasing concentrations (top to bottom) cause increasingly greater depigmentation (photo by Dumont).



Figure 5-11. Eyes Lacking Pigment.

Other eye malformations are frequently associated with depigmentation, as this embryo depicts (photo by Hull).

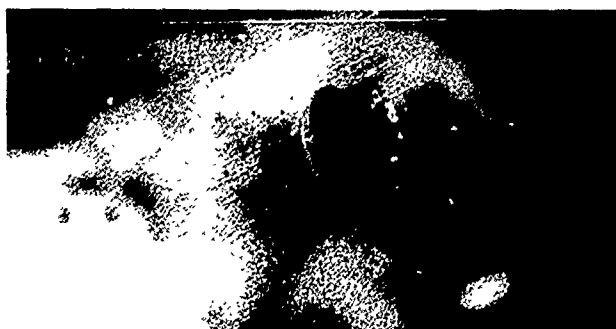


Figure 5-12. Irregular Pigmentation of the Eye.

Pigmentation of the eye and the surrounding area is frequently aberrant. This is typified by irregular eye shape or eccentric accumulations of pigment as is shown in this severely deformed embryo (photo by Hull).



Figure 5-13. Irregular Distribution of Pigment Around the Eye.

Failure of the optic cup to expand and develop will cause a variable "capping" of the eye with pigment. This may be an extreme case of the failure of the choroid fissure to close. This figure shows an external view of the same type of eye malformation that Figure 5-14 shows in cross section (photo by Hull).



Figure 5-14. Cross Section Through the Optic Cup Region of the Eye.

This is a histological cross section through the optic region illustrating abnormal development of the eye. The optic structures are still in direct communication with the ventricles of the brain. The lens, while normal in appearance, has not developed in a normal relationship with other optic tissues. Compare with Figure 5-2; L=lens, PR=incomplete pigmented retina (photo by Dumont).



Figure 5-15. Complete Failure of Eye Development and Retinal Pigmentation.

In this embryo, the pigmentation surrounding the developing eye (arrow) is so sparse that it is difficult to locate those portions of the eye that did develop (photo by Hull).



Figure 5-16. An Eye With Two Lenses.

Sometimes two lenses may develop in the same eye (photo by Hull).



Figure 5-17. Oval Shaped Eyes.

Because of aberrant eye development and pigmentation, the shape and location of the eyes in this embryo is abnormal. Abnormal shape is also frequently associated with edema. The eyes in the edematous optic region of the embryo in this photograph are ovoid - a shape made more obvious by the placement of pigment (photo by Hull).



Figure 5-18. Severely Malformed Eyes.

As shown in this anterior view of a severely abnormal embryo, pigmentation and lateral movement (positioning) of the optic cup has not occurred normally. The development of the brain, head, and face are abnormal and the embryo suffers severe abdominal and cardiac edema (photo by Hull).



Figure 5-19. Displacement of the Eye to a Posterior Location.

This embryo has two anatomically normal eyes. However, one is displaced into the abdominal region (photo by Hull).



Figure 5-20. Narrowing of the Eyes.

Although the eyes of this small embryo appear normal in size, they are located close to the brain and the optic tract is covered with pigment. The next two figures show a progressive narrowing of the eyes towards the midline of the body and cyclopia (photo by Hull).

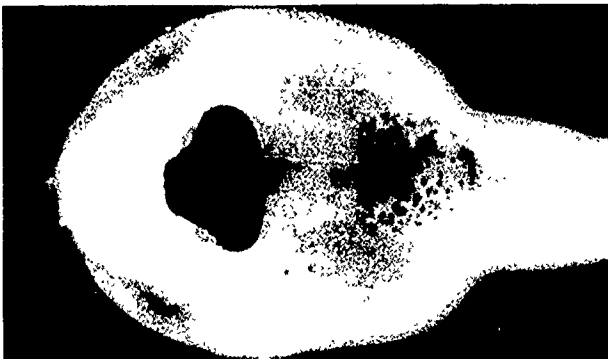


Figure 5-21. Severe Narrowing of the Eyes.

The eyes of this abnormal embryo remain closely associated with the walls of the brain (diencephalon) and pigment covers not only the developing eyes but also the roof of the brain. This condition is not truly cycloptic (photo by Hull).



Figure 5-22. A Cyclops.

A cycloptic embryo with one anatomically correct eye located in the anterior midline (photo by Hull).

Chapter 6

Extreme Head Abnormalities

Brain abnormalities are frequently associated with other gross malformations. The brain normally develops as an expanded tube that forms vesicles through constrictions. These vesicles may increase or decrease in size in response to a toxicant. As the insult increases, the

brain may be rudimentary or completely lacking. In rare cases where the toxicant works at early cleavage, a second embryonic axis may form, leading to a two-headed embryo. In cases where the malformation is less severe, "capping" of the head with pigmented cells is apparent.



Figure 6-1. Reduced Forebrain with "Capping" of Pigment Cells.

Although this embryo shows numerous abnormalities (length, gut, face and head), especially noticeable is the abnormal forebrain. Note that it is abnormally arched over the eyes and deflected downward into the brow area; B=brain (photo by Dumont).



Figure 6-2. Reduced Brain Size Accompanied by a Failure to Form Brain Vesicles.

This is an extremely abnormal embryo with a highly arched brow. The cranium contains a small abnormal brain. This embryo also shows severe head and facial, tail and tail fin, eye, and gut abnormalities. In addition, the embryo is unusually short; B=brain (photo by Hull).

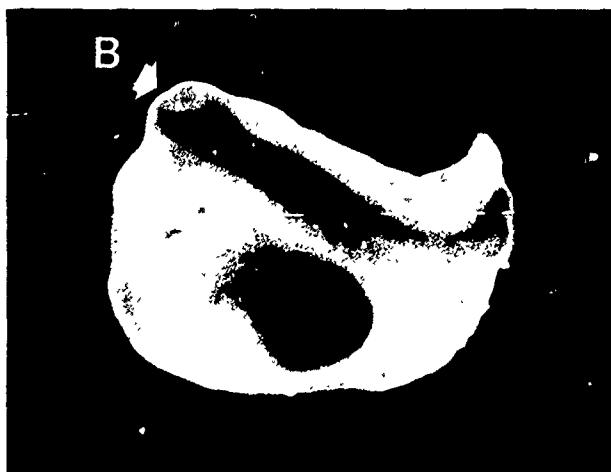


Figure 6-3. Complete Failure of the Brain to Develop.

The neural tube of this embryo has failed to fuse during neurulation. Consequently, this embryo has no brain or eyes but was still alive at the conclusion of the test. All other organs are malformed as well; B=brain (photo by Dumont).

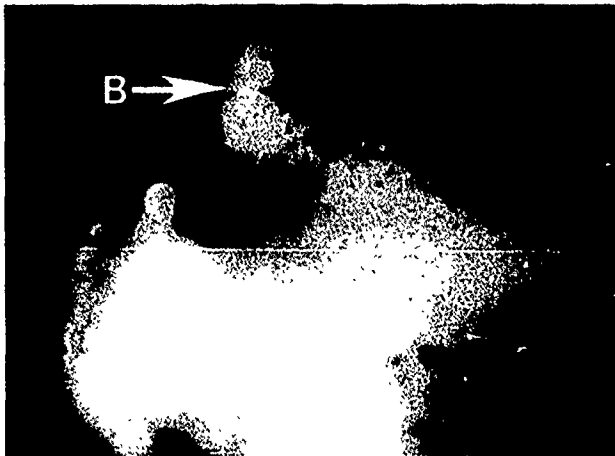


Figure 6-4. An Expanded Brain Vesicle.

This embryo has an expanded brain vesicle that has caused the head to form a peak. Anterior structures are lacking or malformed. The choroid fissure has failed to fuse and the mouth is severely deformed; B=brain (photo by Hull).

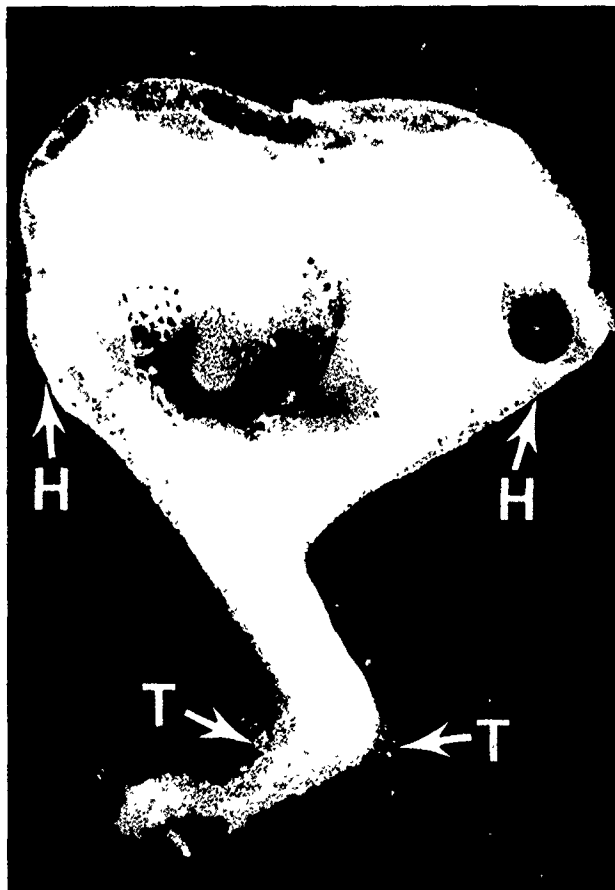


Figure 6-5. Ventral View of a Two-Headed Two-Tailed Embryo

This is a rare malformation that occurs when a severe toxic insult occurs very early in development. Two heads and two tails are clearly evident in this embryo that has established a second longitudinal axis; H=head, T=tail (photo by Hull).

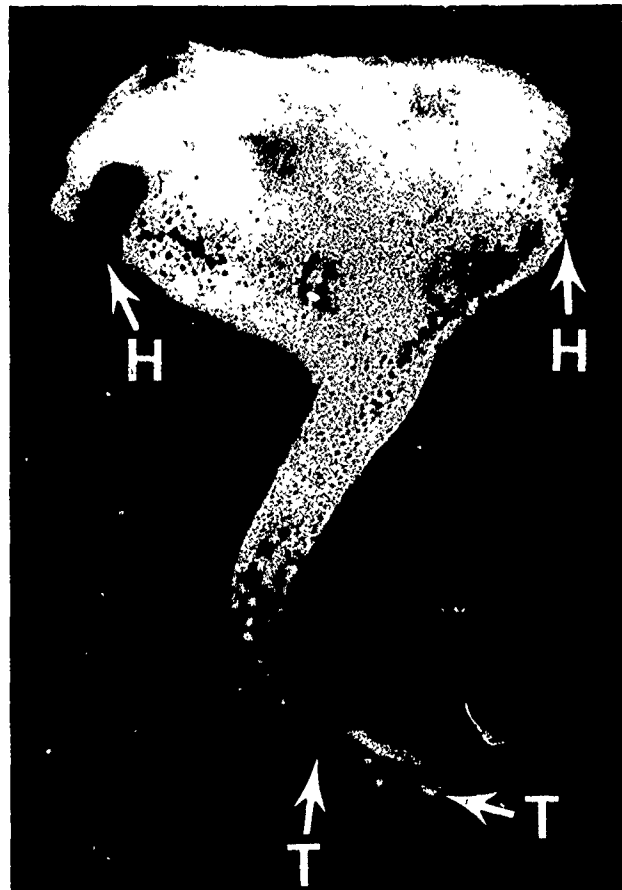


Figure 6-6. Dorsal View of a Two-Headed Two-Tailed Embryo

All organ systems are abnormal; H=head, T=tail (photo by Hull).

Chapter 7

Head and Facial Malformations

Abnormalities of the head and face may be less obvious than other types of malformations and require somewhat more careful examination. Facial abnormalities generally, though not always, appear in conjunction with edema of the eyes, head, or face. Examples of specific head and facial abnormalities are included in the following section. Malformations in unstained embryos may be

difficult for the novice to discern. Until proficiency is gained, the beginning technician should use stained specimens (Appendix III). The tail of the embryo can be inserted into insect screening to hold the face toward the dissection microscope. Often, malformations of the head and brain are accompanied by other severe embryo terata.



Figure 7-1. Scanning Electron Micrograph of the Face of a Normal Stage 46 (96-hr) Embryo.

This scanning electron micrograph showing the facial region of a normal embryo is presented to assist in understanding some of the less obvious features of malformation of the head and face. The metal shadowed eyes appear as lateral bulges. Note the large mouth, with the developing tentacles in the corners, optic bulge, nares surrounded by clumps of ciliated cells, and the oral sucker; M=mouth, O=optic bulge, N=nares, and S=oral sucker (photo by Dumont).

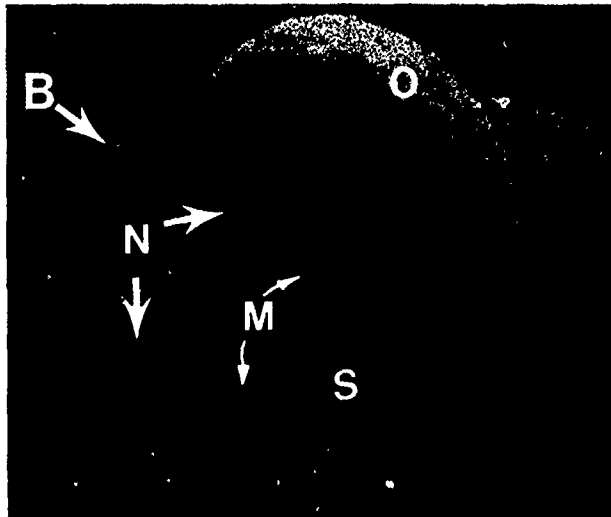


Figure 7-2. Scanning Electron Micrograph of the Face of an Abnormal Embryo.

This scanning electron micrograph shows the head of an embryo with abnormal facial development. The developing nares appear normal. However, optic edema is indicated by the large optic "bulge", the mouth is smaller than normal and the tentacles have not appeared. The "chin" area is extremely narrow suggesting abnormal jaw (cartilage) and oral sucker development. The "brow" region is abnormally high suggesting abnormalities of the brain; B=brow, M=mouth, O=optic bulge, N=nares, and S=oral sucker (photo by Dumont).



Figure 7-3. Light Micrograph of the Face of a Normal Stage 46 (96-hr) Embryo that has been Lightly Stained.

Compare Figure 7-1 with the structures seen here (photo by Hull).

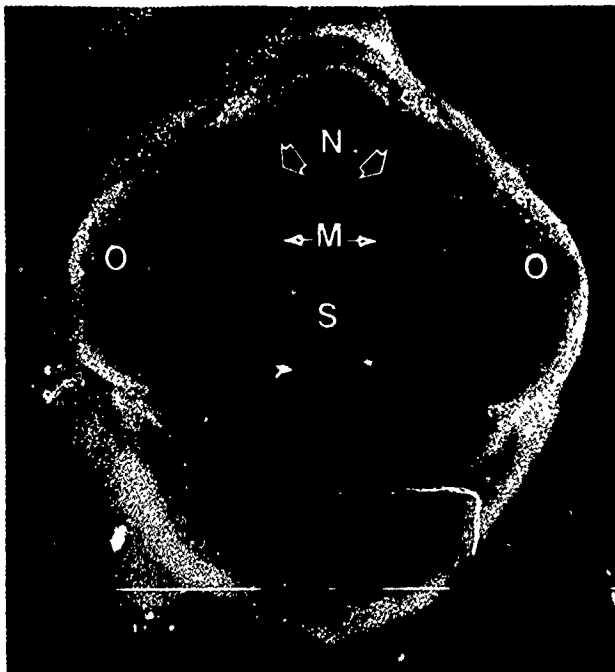


Figure 7-4. Light Micrograph of the Face of an Abnormal Embryo.

Compare the shape and location of facial structures seen here with Figures 7-1 and 7-3. The staining clearly shows radical differences in the placement of the mouth and nares in relation to the eyes. These differences are not readily apparent in unstained embryos; M=mouth, O=optic bulge, N=nares, and S=the oral sucker (photo by Hull).

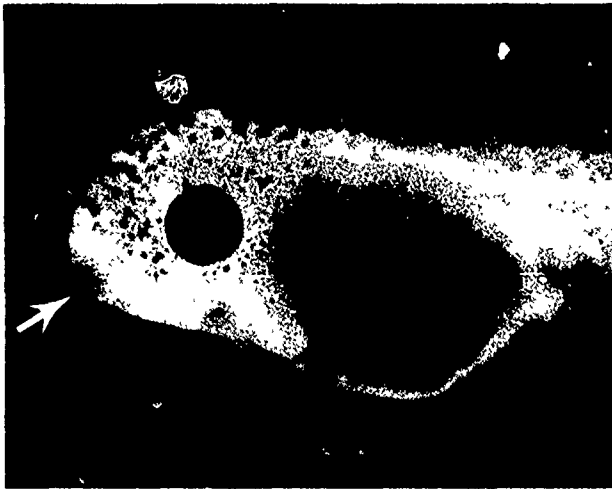


Figure 7-5. A Round Headed Embryo with Facial Defects.

An abnormally small rounded head and malformed facial region is displayed by this embryo. In many cases where the head is small (microcephaly), the oral sucker (arrow) appears prominent, suggesting that in such cases the development of this organ is unaffected. The eyes, although somewhat smaller than normal, appear proportional to the size of the head. In addition, the embryo itself is abnormally short. Gut development is characterized by a lack of appropriate coiling (photo by Dumont).



Figure 7-6. Abnormal Embryo with Prominent Forehead and Extended Mouth.

Abnormal head and facial development is depicted by the flattened face and rounded brow. The eye appears normal. There is slight cardiac edema around the heart and inadequate gut coiling, considering that this embryo is 96 hours old. The oral sucker appears prominent; H=heart and S=oral sucker (photo by Rayburn).

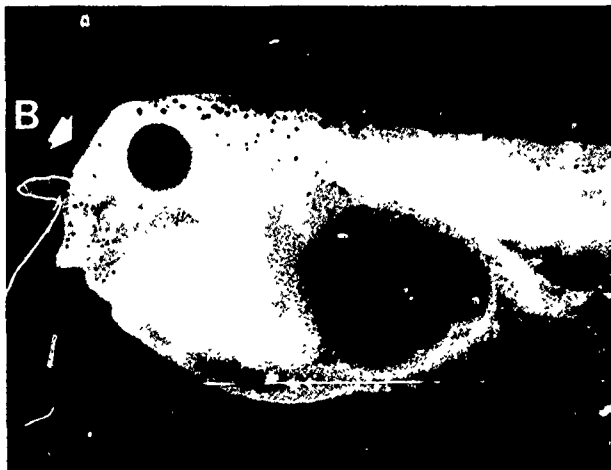


Figure 7-7. An Embryo with a Sloping Forehead and Distended Mouth.

Despite the apparently normal gut and eye, the head and face region of this embryo is significantly malformed. The face is extremely flattened and the forebrain is deflected downward along the front of the brow; B=brain (photo by Rayburn).

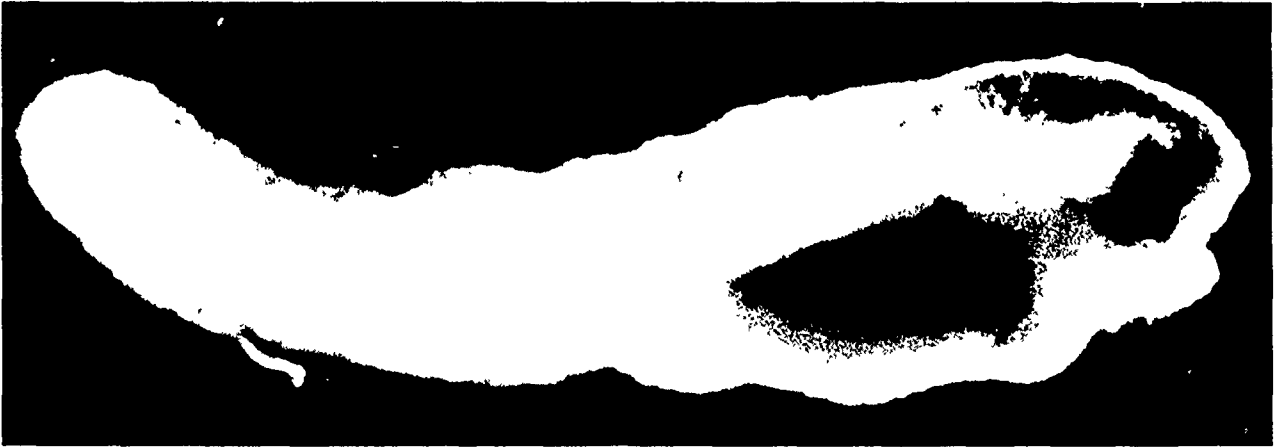


Figure 7-8. An Embryo with a Severely Malformed Head and Face.

This embryo has severe malformations in the gut and tail as well as abnormal pigmentation. Despite the small size of the head, quantitative histological data (Dumont, unpublished) indicate the brain ventricles (vesicles) are actually enlarged (hydrocephaly) (photo by Hull).

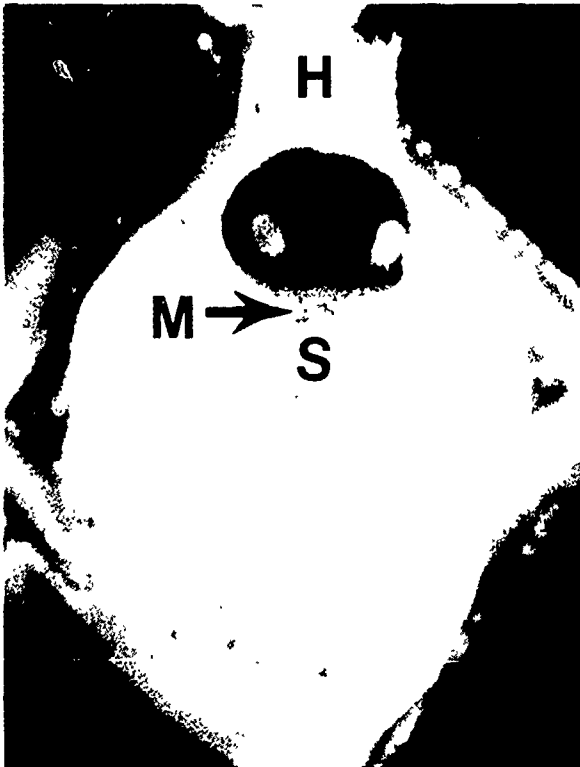


Figure 7-9. Facial View of an Embryo with a Severely Reduced Head and Face.

Note that the brain ventricles (vesicles) in this embryo are poorly developed and the head is reduced to a mound of tissue. The two eyes with lenses are fused. The mouth is greatly reduced in size and the oral sucker appears as an oval, M=mouth, H=head and S=oral sucker (photo by Hull).

Chapter 8

Heart Malformations

The detection of cardiac malformations in Stage 46 (96-hr) embryos is difficult for the novice. After formalin fixation, the heart, which is visible through the transparent skin, becomes opaque and its development is difficult to assess. It is best to learn to detect malformations in anesthetized or stained animals before identifying them in formalin-fixed embryos. Appendix III gives information on procedures for anesthetizing and staining Stage 46 embryos.

It should be remembered that the heart begins development as a pair of tubes that come together to form a single, independently beating tube that works by peristaltic action. It develops a loop and then vesicles are

formed by expansion. These vesicles become the auricles and the ventricle. Developmental toxicants may slow or alter this process leaving only a short, muscular tube to sustain life by peristaltic action. A beating heart is an unambiguous sign of life even though the heart may not be properly formed. When the heart is slowly beating following anesthesia, it is easy to observe abnormalities as the heart pumps blood (see Figures 8-1 and 8-2). As proficiency is gained, abnormal embryos can be stained and observed for defects. After the investigator is competent with stained embryos, malformed hearts may be detected more readily in formalin-fixed embryos.

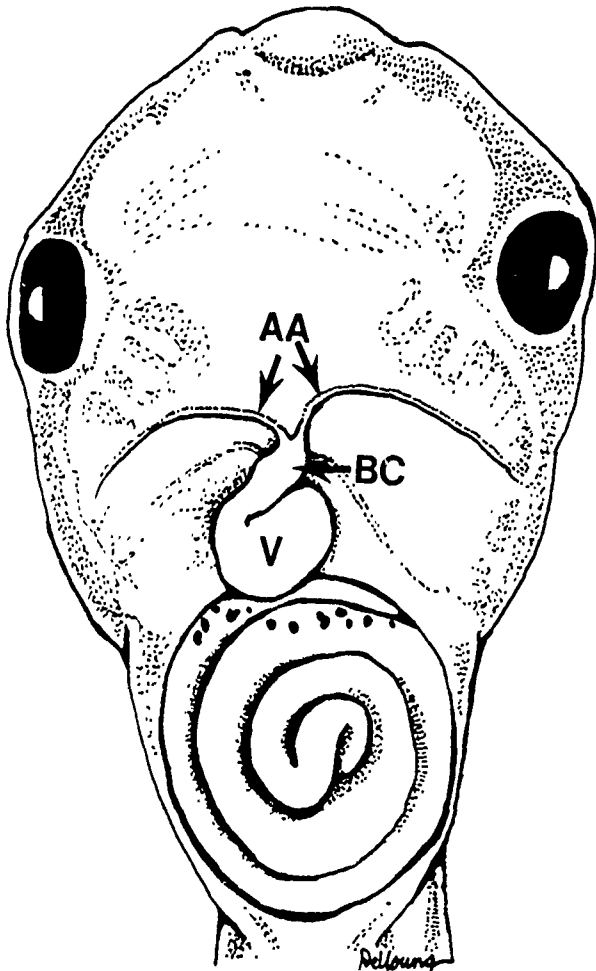


Figure 8-1. Diagram of a Normal Stage 46 (96-hr) Embryo with Normal Heart and Gut (Ventral View).

The shape of the vesicles vary as the heart beats as seen in Figure 8-2. Fixation moves the vesicles into slightly different positions. Note the aortic arches that lead away from the heart; AA=aortic arches, BC=bulbous cordis, V=ventricle of the heart; the atria are immediately above the ventricle (diagram by DeYoung).

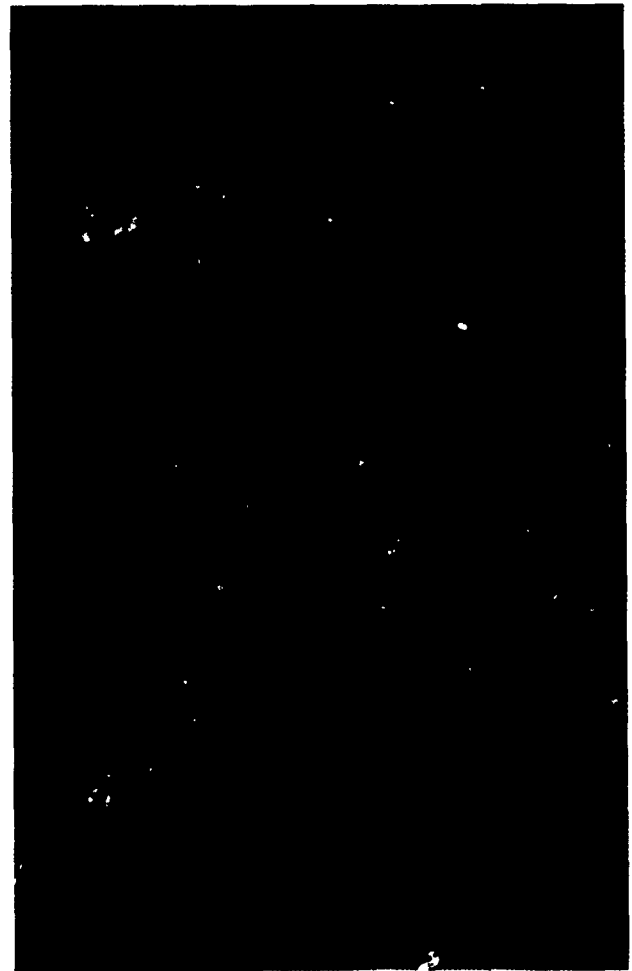


Figure 8-2. MS-222 Anesthetized Normal Stage 46 (96-hr) Embryo (Ventral View).

Note the red blood in the large ventricle of this live specimen. The blood can be followed as it goes from one chamber to another (photo by Work).



Figure 8-3. Stained Normal Stage 46 (96-hr) Embryo (Ventral View).

The staining procedure used makes it much easier to observe the heart and aortic arches. Compare this figure with Figures 8-2 and 8-4 (photo by Hull).

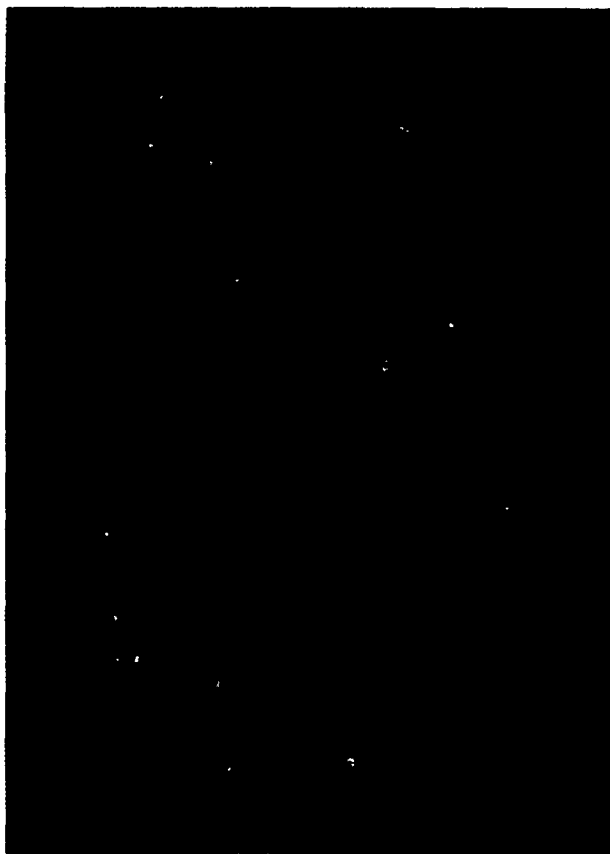


Figure 8-4. Formalin Fixed Normal Stage 46 (96-hr) Embryo (Ventral View).

The 3% v/v formalin fixation renders the heart as a translucent mass of tissue above the coiled gut. Abnormal embryos are usually examined in formalin so face and heart malformations may be harder to observe. The anesthesia and staining protocols are time consuming and should only be used in training (photo by Hull).

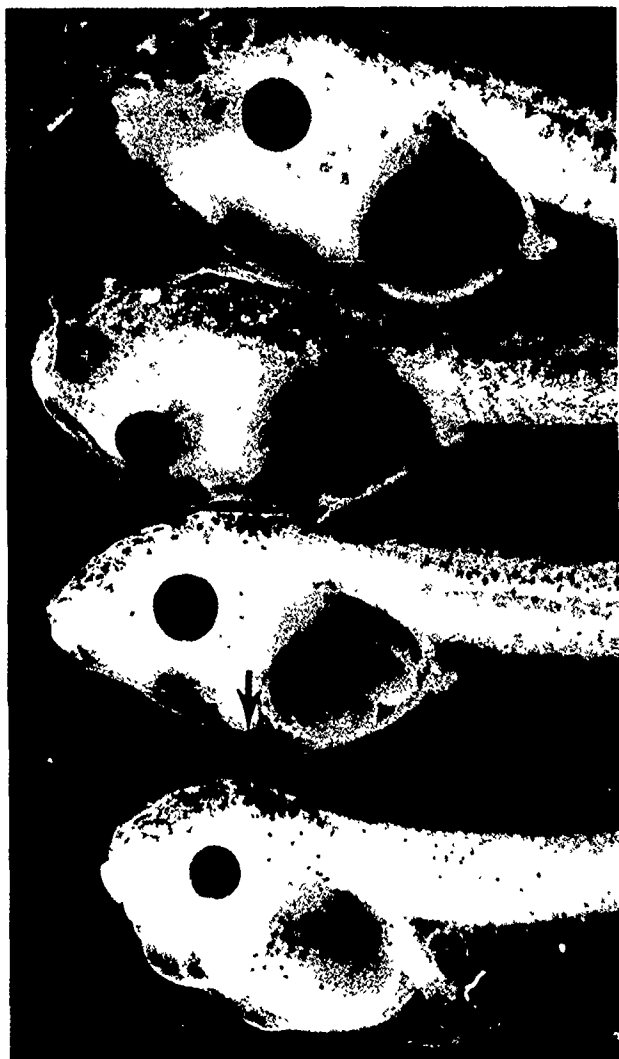


Figure 8-5. Increasing Concentrations of a Developmental Toxicant Result in Increasingly Severe Heart Malformations.

The heart of the control embryo (top) seen in this lateral view is normal. The heart of the embryo that is third from the top shows an abnormal expansion of the ventricle (arrow) just in front of the gut. The bottom embryo shows the heart as a large amorphous mass in an edematous pericardial cavity. These embryos are all formalin-fixed (photo by Hull).



Figure 8-6. Severe Failure of the Heart Tube to Coil Properly.

The Stage 46 embryo at the top is a control and shows a properly coiled heart in lateral view. The embryo at the bottom shows a displaced heart with abnormal vesicles in front of the gut (arrow) (photo by Hull).



Figure 8-7. A Poorly Coiled Heart and Pericardial Edema.

The top embryo (control) shows a properly coiled heart from a right lateral view. The abnormal embryo below has a straight tube (arrow) in some places (near gut) and an anterior bulging vesicle. The severe pericardial edema has pushed the heart into the dorsal portion of the body cavity; H=heart and V=vesicle (photo by Hull).

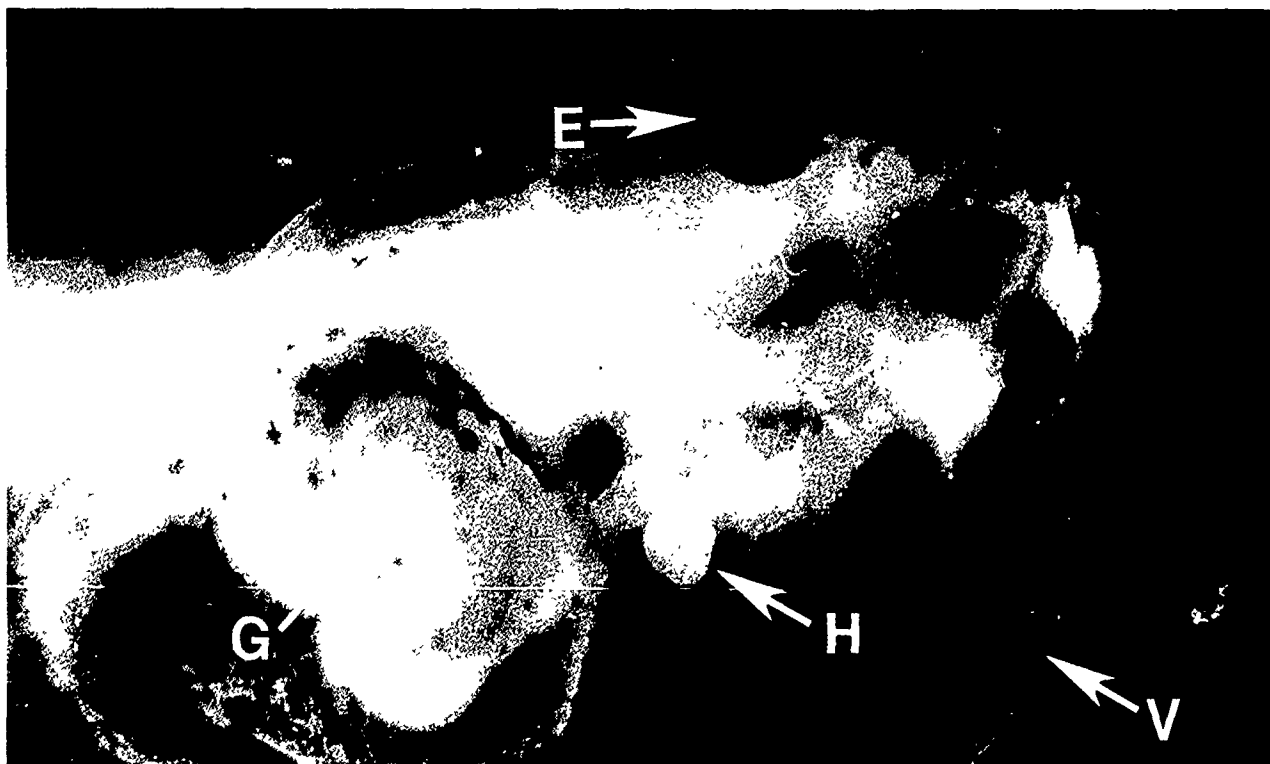


Figure 8-8. Displaced Heart with Abnormal Vesicle Formation.

This is a severely deformed Stage 46 (96-hr) embryo with pericardial edema. The vesicles have formed as several prominent bulges that are out of position. The pericardial area has swollen away from the heart; H=heart, G=gut, E=eye and V=vesicle (photo by Hull).



Figure 8-9. A Heart Composed of a Single Vesicle and a Straight Tube.

A very severe malformation (arrow) is easily detected in this formalin-fixed Stage 46 (96-hr) embryo. The edema made detection easy (photo by Hull).

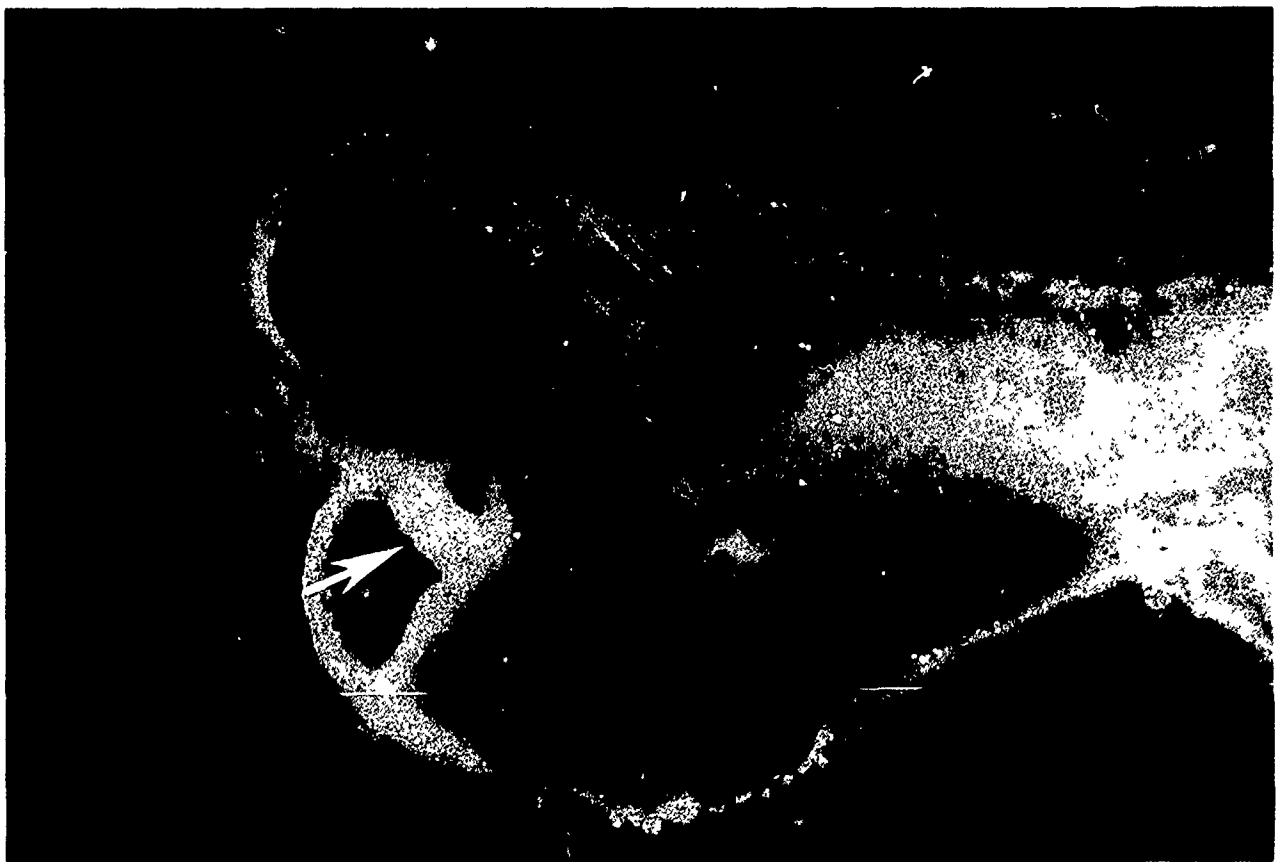


Figure 8-10. A Heart Composed of a Single Straight Tube.

A heart composed of a single beating tube (arrow) (photo by Hull).

Chapter 9

Gut Abnormalities

Abnormal development of the gastrointestinal tract (gut) is characterized primarily by the lack of coiling. Coiling begins at Stage 39 (56-hr) and by Stage 46 (96-hr) the gut has normally undergone two to two and one half coiling revolutions (Niekoop and Faber, 1975). Failure of the gut to coil by Stage 46 may be due to either abnormal development or developmental delay. If the malformations appear slight, it might be desirable to let some of the embryos continue to develop past 96 hours in clean FETAX

solution. If the gut finally coils properly then it is not an abnormal embryo but a case of developmental delay. Developmental delay is an important measurement which can be recorded separately. Although the gut shows extremely well in formalin-fixed embryos, the detection of abnormal gut coiling is made more difficult by cases of developmental delay. Edema in the abdomen is suggestive of abnormal development.

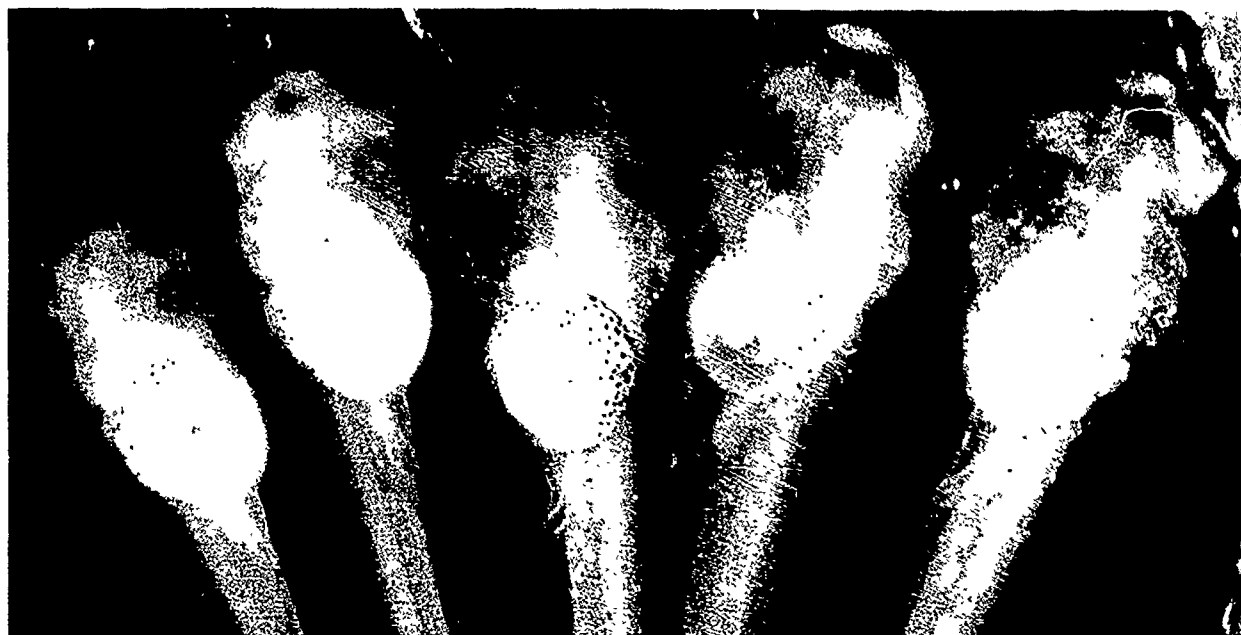


Figure 9-1. Various Stages in Normal Gut Development.

Control embryos were fixed in formalin after various times of normal development. After fixation they were arranged to show the normal coiling of the gut. From left to right the hours of development are. 76 hr, 80 hr, 92 hr, 96 hr, 100 hr. All these embryos were incubated at 23.5 °C (photo by Hull).

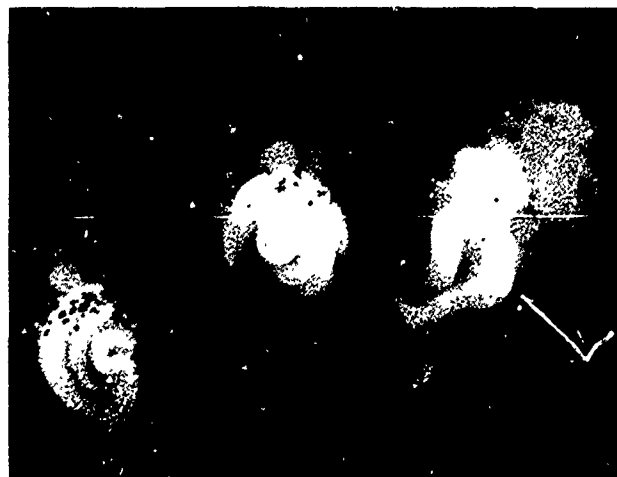


Figure 9-2. Various Degrees of Incomplete Gut Coiling Caused by Toxicant Action.

Compare with Figure 9-1 to see how closely these abnormal guts may resemble earlier stages of gut development (photo by Hull).



Figure 9-3. Relationship of Gut Coiling to Toxicant Concentration.

Here extreme gut malformation is shown in moderate concentrations of teratogen (control at top). The second embryo, while possessing some subtle facial and head malformations, has a normal appearing gut. The third embryo, however, has a looser coiled gut while the embryo at the bottom of the photograph has severe abnormalities, including a malformed gut. In extreme cases, the gut may be a simple straight tube that never coils properly. Note the blisters along dorsal midline above the abdomen, severe edema, short length, and malformed head and eyes in the bottom embryo (photo by Rayburn).



Figure 9-4. Relationship of Gut Coiling to Toxicant Concentration.

In cases where edema is not a part of the syndrome, the developing gut remains more-or-less compact. Its lack of coiling is easily detected. Note the abnormal heads and short length. The highest concentration is on the left and decreases toward the right. The control is the last embryo on the right (photo by Hull).



Figure 9-5. Relationship of Gut Coiling to Toxicant Concentration.

Shown is a series of embryos (control at top) exposed to increasing concentrations of a teratogen. Progressive malformation (lack of coiling) of the gut can be seen as the concentration increases. In the second and third embryo, the gut is coiled but not to the extent normally expected by Stage 46. In the fourth embryo, coiling has not occurred and the gut appears sigmoid while in the bottom embryo, the gut is essentially an enlarged straight tube. In severe cases, the yolk will leak into the gut and anus. Other progressively severe abnormalities include those of the head and face, brain, face, and tail (photo by Dumont).

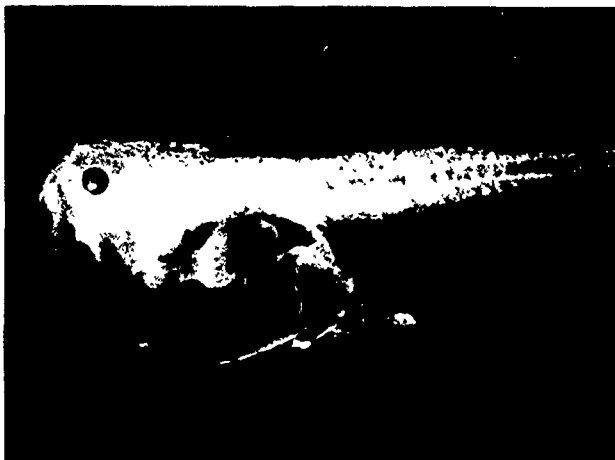


Figure 9-6. Complete Lack of Coiling of the Gut.

When edema is present, malformations of the gut are easier to detect. In this case, the gut has coiled into a single loop instead of coiling normally. Note severe edema in the abdominal, cardiac, and optic regions. The eye is reduced in size and is poorly pigmented (photo by Dumont).

Appendix I
FETAX Data Sheets

FETAX SUMMARY SHEET

| | | |
|---|-------------------------|---------------------------------------|
| Test Material <u>13-cis-Retinoic Acid</u> | | Test No. <u>4</u> |
| Source <u>Sigma</u> | | Investigator <u>D. Young</u> |
| CAS No. <u>4759-48-2</u> | Lot No. <u>58-F0681</u> | Lab <u>BanHe/OSU</u> |
| Composition/Purity <u>99%</u> | | Test Start Date <u>1-24-90</u> |
| Solvent <u>none</u> | Conc. <u>n/a</u> | Test End Date <u>1-28-90</u> |
| | | Test Units (i.e., mg/ml) <u>ng/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | 7.2 | 7.2 | 7.2 | 7.2 | |
| Control | 7.1 | 7.0 | 7.4 | 7.3 | |
| Highest Conc. | 7.3 | 7.0 | 7.1 | 7.2 | |

| FETAX CONTROL | MORTALITY RECORD | MALFORMATION RECORD |
|--|--|---|
| No. Dead or Malformed Total Number X 100 = % | | |
| | <u>2</u> : <u>100</u> X 100 = <u>2</u> % | <u>100</u> : <u>98</u> X 100 = <u>1</u> % |
| Solvent Control <u>none</u> | <u>-</u> : <u>-</u> X 100 = <u>-</u> % | <u>-</u> : <u>-</u> X 100 = <u>-</u> % |
| Control Length <u>8.99</u> mm | Solvent Control Length <u>-</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>10</u> | | |

TEST MATERIAL/COMPOUND : RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|-------------|--|--------------------------|
| NOEL | <u>10</u> | <u>1</u> | <u>Bonferroni T-test</u> |
| LOEL | <u>30</u> | <u>2</u> | <u>Bonferroni T-test</u> |
| LC ₅₀ | <u>35.7</u> | EC ₅₀ | <u>3.5</u> |
| 95% Confidence limits <u>(34.1 - 37.4)</u> | | 95% Confidence Limits <u>(3.1 - 4.0)</u> | |
| TEST TERATOGENIC INDEX (TI = LC ₅₀ / EC ₅₀) | | | <u>10.1</u> |

POSITIVE CONTROL: 6 AMINONICOTINAMIDE (6-AN) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|---------------|---|--|
| 5.5 mg/L | <u>2</u> : <u>50</u> X 100 = <u>4</u> % | <u>23</u> : <u>48</u> X 100 = <u>48</u> % |
| 2500 mg/L | <u>32</u> : <u>50</u> X 100 = <u>64</u> % | <u>18</u> : <u>18</u> X 100 = <u>100</u> % |

FETAX SUMMARY SHEET

| | | |
|--------------------|---------|--------------------------|
| | | Test No. |
| Test Material | | Investigator |
| Source | | Lab |
| CAS No. | Lot No. | Test Start Date |
| Composition/Purity | | Test End Date |
| Solvent | Conc. | Test Units (i.e., mg/ml) |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| -- pH -- | | | | | |
| Lock | | | | | |
| Control | | | | | |
| Highest Conc. | | | | | |

| FETAX CONTROL | MORTALITY RECORD | MALFORMATION RECORD |
|--|-------------------------------|-------------------------------|
| No. Dead or Malformed | | |
| _____ X 100 = % | | |
| Total Number | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Solvent Control | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Control Length mm | Solvent Control Length mm | |
| Minimum Concentration to Inhibit Growth (MCIG) | | |

TEST MATERIAL/COMPOUND : RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------|-----------------------|--------------|-----------------------|
| NOEL | | | |
| LOEL | | | |
| LC ₅₀ | EC ₅₀ | | |
| 95% Confidence limits | 95% Confidence Limits | | |

TEST TERATOGENIC INDEX (TI = LC₅₀ / EC₅₀)

POSITIVE CONTROL: 6 AMINONICOTINAMIDE (6-AN) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|---------------|-------------------------------|-------------------------------|
| 5.5 mg/L | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| 2500 mg/L | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |

| | |
|---------------|--------------------------------|
| Investigator | D. DeYoung |
| Date | 1-29-90 |
| Test Material | 13-cis-Retinoic Acid 4759-48-2 |
| Test Number | 4 |

* 275 ng/ml stock concentration

[illegible]

| | |
|---------------------|----------------------|
| Investigator | Test Material |
| Date | Test Number |

FETAX MALFORMATION DATA

| | | | |
|--------------|------------|---------------|----------------------|
| Investigator | D. DeYoung | Test Material | 13-cis-Retinoic Acid |
| Date | 1-29-90 | Test Number | 4 |

| MALFORMATION | CONCENTRATION * ng/ml | | | | | | | | | | | | | | TOTAL |
|---------------|-----------------------|-----|-----|-----|-----|-----|----|----|----|----|----|----|--|--|-------|
| | C | 0.9 | 1.0 | 2.0 | 3.0 | 5.0 | 10 | 20 | 30 | 40 | 50 | 55 | | | |
| Severe | 1 | | | 1 | 3 | | 49 | 46 | 41 | 14 | 3 | 4 | | | 162 |
| Stunted | | | | | | | | | | | | | | | |
| Gut | | 1 | 1 | 1 | 1 | 1 | | | | | | | | | 5 |
| Edema | | | | | | | | | | | | | | | |
| Multiple | | | | | | | | | | | | | | | |
| Cardiac | | | 1 | | 2 | | | | | | | | | | 3 |
| Abdominal | | 1 | | | | | | | | | | | | | 1 |
| Facial | | | | | | | | | | | | | | | |
| Cephalic | | | | | | | | | | | | | | | |
| Optic | | | | | | | | | | | | | | | |
| Tail | | | | 4 | 10 | 18 | | | | | | | | | |
| Notochord | | | | | 11 | 16 | | | | | | | | | |
| Fin | | | | | | | | | | | | | | | |
| Face | | 4 | 8 | 8 | 13 | 35 | | | | | | | | | 68 |
| Eye | | | 2 | 1 | | | | | | | | | | | 3 |
| Brain | | | 2 | 1 | | | | | | | | | | | 3 |
| Hemorrhage | | | | | | | | | | | | | | | |
| Cardiac | | | 1 | 1 | | | | | | | | | | | 2 |
| Blisters | | | | | | | | | | | | | | | |
| Other-specify | | | | 1 | 9 | 14 | | | | | | | | | 24 |
| No. Malformed | 1 | 2 | 3 | 10 | 15 | 35 | 49 | 46 | 41 | 14 | 3 | 4 | | | X |
| Total No. | 98 | 49 | 50 | 50 | 49 | 50 | 49 | 46 | 41 | 14 | 3 | 4 | | | 372 |

Comments:

FETAX MALFORMATION DATA

| | |
|--------------|---------------|
| Investigator | Test Material |
| Date | Test Number |

| MALFORMATION | CONCENTRATION | | | | | | | | | | | | | | | | | | | | TOTAL |
|---------------|---------------|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|-------|
| | | | | | | | | | | | | | | | | | | | | | |
| Severe | | | | | | | | | | | | | | | | | | | | | |
| Stunted | | | | | | | | | | | | | | | | | | | | | |
| Gut | | | | | | | | | | | | | | | | | | | | | |
| Edema | | | | | | | | | | | | | | | | | | | | | |
| Multiple | | | | | | | | | | | | | | | | | | | | | |
| Cardiac | | | | | | | | | | | | | | | | | | | | | |
| Abdominal | | | | | | | | | | | | | | | | | | | | | |
| Facial | | | | | | | | | | | | | | | | | | | | | |
| Cephalic | | | | | | | | | | | | | | | | | | | | | |
| Optic | | | | | | | | | | | | | | | | | | | | | |
| Tail | | | | | | | | | | | | | | | | | | | | | |
| Notochord | | | | | | | | | | | | | | | | | | | | | |
| Fin | | | | | | | | | | | | | | | | | | | | | |
| Face | | | | | | | | | | | | | | | | | | | | | |
| Eye | | | | | | | | | | | | | | | | | | | | | |
| Brain | | | | | | | | | | | | | | | | | | | | | |
| Hemorrhage | | | | | | | | | | | | | | | | | | | | | |
| Cardiac | | | | | | | | | | | | | | | | | | | | | |
| Blisters | | | | | | | | | | | | | | | | | | | | | |
| Other-specify | | | | | | | | | | | | | | | | | | | | | |
| No. Malformed | | | | | | | | | | | | | | | | | | | | | |
| Total No. | | | | | | | | | | | | | | | | | | | | | |

Comments:

FETAX MALFORMATION DATA

| | | | |
|---------------|----------------------|--------------|------------|
| Test Material | 13-cis-Retinoic Acid | Investigator | D. DeYoung |
| Test No. | 4 | Date | 2-1-90 |

[illegible]

FETAX MALFORMATION DATA

| | |
|---------------|--------------|
| Test Material | Investigator |
| Test No. | Date |

[illegible]

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | | |
|---------------|----------------------|----------------|-----------|
| Test Material | 13-cis Retinoic Acid | CAS No. | 4759-48-2 |
| Test No. | 4 | Vendor/Lot No. | 58-F0631 |

[illegible]

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | |
|---------------|----------------|
| Test Material | CAS No. |
| Test No. | Vendor/Lot No. |

[illegible]

FETAX with METABOLIC ACTIVATION (MA) Summary Sheet

| | |
|---------------------------------|---------------------------------------|
| Test No. 3 | |
| Test Material Rifampicin | Investigator D. Fort |
| Source Sigma | Lab Bantle / OSU |
| CAS No. 13292-46-1 | Lot No. 47-R254 |
| Composition/Purity 99% | Test Start Date 3/2/90 |
| Solvent DMSO | Test End Date 3/6/90 |
| Conc. 1% V/V | Test Units (i.e., mg/ml) ng/ml |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 6.9 | 7.0 | 7.1 | 7.0 | |
| Control | 7.0 | 7.3 | 7.4 | 7.4 | |
| Highest Conc. | 7.1 | 7.4 | 7.7 | 7.4 | |

| FETAX CONTROL | MORTALITY RECORD | MALFORMATION RECORD |
|--|-----------------------|----------------------|
| No. Dead or Malformed Total Number X 100 = % | | |
| Solvent Control | 0 : 80 X 100 = 0 % | 0 : 80 X 100 = 0 % |
| Metabolic Activation Control | 0 : 40 X 100 = 0 % | 1 : 40 X 100 = 2.5 % |
| Metabolic Activation System plus Solvent Control | 0 : 40 X 100 = 0 % | 2 : 40 X 100 = 5 % |
| Cyclophosphamide Control (4.0 mg/ml) | 40 : 40 X 100 = 100 % | — : — X 100 = — % |
| CO-Metabolic Activation System plus Test Material | 0 : 40 X 100 = 0 % | 4 : 40 X 100 = 10 % |
| MA + Solvent Control Length 7.99 mm | | |
| Minimum Concentration to Inhibit Growth (MCIG) 0.25 | | |

TEST MATERIAL RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|-------------|--|-----------------------|
| NOEL | not done | not done | |
| LOEL | not done | not done | |
| LC ₅₀ | 1.35 | EC ₅₀ 0.40 | |
| 95% Confidence limits (1.26 - 1.46) | | 95% Confidence Limits (0.39 - 0.55) | |
| TEST TERATOGENIC INDEX (TI = LC ₅₀ / EC ₅₀) | | | 2.89 |

FETAX with METABOLIC ACTIVATION (MA) Summary Sheet

| | | |
|--------------------|---------|--------------------------|
| | | Test No. |
| Test Material | | Investigator |
| Source | | Lab |
| CAS No. | Lot No. | Test Start Date |
| Composition/Purity | | Test End Date |
| Solvent | Conc. | Test Units (i.e., mg/ml) |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | | | | | |
| Control | | | | | |
| Highest Conc. | | | | | |

| FETAX CONTROL | MORTALITY RECORD | MALFORMATION RECORD |
|--|-------------------------------|-------------------------------|
| No. Dead or Malformed | | |
| Total Number | | |
| X 100 = % | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Solvent Control | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Metabolic Activation Control | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Metabolic Activation System plus Solvent Control | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| Cyclophosphamide Control (4.0 mg/ml) | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| CO-Metabolic Activation System plus Test Material | _____ : _____ X 100 = _____ % | _____ : _____ X 100 = _____ % |
| MA + Solvent Control Length mm | | |
| Minimum Concentration to Inhibit Growth (MCIG) | | |

TEST MATERIAL RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------|-----------|-----------------------|-----------------------|
| NOEL | | | |
| LOEL | | | |
| LC ₅₀ | | EC ₅₀ | |
| 95% Confidence limits | | 95% Confidence Limits | |

TEST TERATOGENIC INDEX (TI = LC₅₀ / EC₅₀)

Appendix II
Chemical Exposure Information

Table I. Test Material and Exposure Data.*

Chapter 3 - Axial Malformations

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description of Malformations</u> |
|-------------|---|----------------------|--|
| 1 | acetazolamide | 0.001 mg/ml | simple dorsal curve |
| 2 | acetazolamide w/penicillin & streptomycin | 0.12 mg/ml | moderate ventral flexure |
| 3 | acetazolamide w/penicillin & streptomycin | 0.12 mg/ml | lateral flexure |
| 4 | semicarbazide | 200 mg/l | severe lateral flexures |
| 5 | (CRM-2)(aqueous extract of crude shale oil) | 7.5 % | dorsal tail flexure |
| 6 | shale crude | 10% crude oil | curved tails |
| 7 | captan | C**, 1.3 mg/l | wavey tail |
| 8 | acetic acid hydrazide | C, 0.06, 12 mg/ml | severe wavey tail |
| 9 | thiosemicarbazide | 100 mg/l | scanning electron micrograph of a tail section |
| 10 | salicylic acid | 1mg/ml, C | axial shortening |

Chapter 4 - Edema

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description of Malformations</u> |
|-------------|--|-----------------------|---|
| 1 | lithium carbonate | 40 mg/l | moderate blistering |
| 2 | CRM-4 (electrostatic precipitator tar) | 0.5% | scanning electron micrograph w/blistering |
| 3 | meclizine | C, 5, 25 mg/l | cardiac edema |
| 4 | aminopyrene | C, 100, 300, 700 mg/l | abdominal edema |
| 5 | none | control | mild optic edema |
| 6 | trichloroethylene | 0.001% v/v | severe optic edema |
| 7 | shale oil crude extract | 10% | histological section of head |
| 8 | none | control | anterior view w/edema |
| 9 | methyl arsenate | 25 mg/l | severe generalized edema |

Chapter 5 - Eye

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description of Malformations</u> |
|-------------|---|----------------------|--|
| 1 | none | control | normal Stage 46 (96-hr) eye |
| 2 | none | control | histological section of Stage 46 (96-hr) eye |
| 3 | acetazolamide w/penicillin & streptomycin | 0.15 mg/ml | reduced eyes w/edema |
| 4 | none | control | small eye w/normal eye |
| 5 | acetazolamide | 0.01 mg/ml | choroid fissure failure |
| 6 | none | control | moderate failure of choroid |
| 7 | none | control | severe failure of choroid |
| 8 | acetazolamide w/penicillin & streptomycin | 0.15 mg/ml | optic cup rupture |
| 9 | 6-aminonicotinamide | 0.004 mg/ml | severe optic cup rupture |
| 10 | ethylene thiourea | range unknown | progressive de-pigmentation |
| 11 | unknown groundwater sample | 25% concentration | eyes lacking pigmentation |
| 12 | none | control | irregular pigmentation |
| 13 | none | control | irregular pigment distribution |
| 14 | methanol | 3.2% | cross section with incompletely pigmented retina |
| 15 | retinoic acid | 0.3 µg/ml | failure of pigment to deposit |
| 16 | 13-cis retinoic acid | 0.04 µg/ml | two lenses |
| 17 | hydroxyurea with jelly coat | 0.9 mg/ml | oval-shaped eyes |
| 18 | aspartame | 8.0 mg/ml | severely malformed eyes |
| 19 | acetazolamide | 0.5 mg/ml | eye displaced caudad |
| 20 | none | control | narrowing of the eyes |
| 21 | acetazolamide w/penicillin & streptomycin | 0.07 mg/ml | severe narrowing of the eyes |
| 22 | 13-cis retinoic acid | 0.3 ng/ml | a true cyclops |

Chapter 6 - Brain

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description of Malformations</u> |
|-------------|--|----------------------|-------------------------------------|
| 1 | trypan blue | 200 mg/l | reduced forebrain |
| 2 | none | control | reduced brain size |
| 3 | trans-retinoic acid | 0.05 mg/l | failure of the brain to develop |
| 4 | acetazolamide w/penicillin & streptomycin | 0.07 mg/ml | expanded brain vessicle |
| 5 | acetazolamide | 0.001 mg/ml | two-headed two-tailed embryo |
| 6 | acetazolamide | 0.001 mg/ml | two-headed two-tailed embryo |

Chapter 7 - Face

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description of Malformations</u> |
|-------------|--|----------------------|--|
| 1 | none | control | scanning electron micrograph of Stage 46 (96-hr) tadpole |
| 2 | ethanol | 2.5% | scanning electron micrograph of stunted face |
| 3 | none | control | face of Stage 46 (96-hr) tadpole |
| 4 | nickel | 30 mM | abnormal nose, mouth and sucker |
| 5 | pseudoephedrine | 0.03 mg/ml | round headed tadpole |
| 6 | methotrexate | 0.05 mg/ml | tadpole w/prominent forehead |
| 7 | trans-retinoic acid | 0.2 µg/ml | tadpole w/sloping forehead |
| 8 | methotrexate | 100 mg/ml | severely microcephalic |
| 9 | acetazolamide w/penicillin & streptomycin | 0.07 mg/ml | face w/reduced head |

Chapter 8 - Heart

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description</u> |
|-------------|----------------------|--------------------------|--------------------------------|
| 1 | none | control | drawing |
| 2 | none | control | live heart |
| 3 | none | control | stained heart |
| 4 | acetone | 0.9% v/v | formalin-fixed heart |
| 5 | coumarin | C, 0.07, 0.14, 0.2 mg/ml | increasing heart malformations |
| 6 | isoniazid | C, 5.0 mg/ml | failure of heart to coil |
| 7 | isoniazid | C, 7.5 mg/ml | poorly coiled heart |
| 8 | none | control | displaced heart |
| 9 | isoniazid | 2.0 mg/ml | heart w/single vesicle |
| 10 | isoniazid | 7.5 mg/ml | straight-tube heart |

Chapter 9 - Gut

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description</u> |
|-------------|----------------------|---------------------------------|--|
| 1 | none | control | hourly control's 72, 80, 92, 96, and 100 hours |
| 2 | coumarin | C, 0.16, 0.28 mg/ml | degrees of incomplete gut coiling |
| 3 | trans-retinoic acid | C, 0.02, 0.2, 0.5 µg/ml | gut coiling vs. a toxicant concentration |
| 4 | α-solanine | C, 12, 14, 16 mg/l | gut coiling vs. a toxicant concentration |
| 5 | methotrexate | C, 50, 100, 200, 300, 400 mg/ml | gut coiling vs. a toxicant concentration |
| 6 | dimethylbenzopyrine | 1.0 mg/l | complete lack of gut coiling |

Back Cover

| <u>Fig#</u> | <u>Test Material</u> | <u>Concentration</u> | <u>Description</u> |
|-------------|----------------------|----------------------|--------------------|
| 1 | α-solanine | 7 mg/l | embryo twinning |

*This table shows the test material and the concentration that caused the malformation depicted in the Figure.

**C=control

Appendix III

Miscellaneous Information

The Use of a Metabolic Activation System in FETAX

Introduction

A metabolic activation system (MAS) is only required when FETAX is used to identify possible human health hazards. The MAS is composed of induced rat liver microsomes and an NADPH generator system which simulates mammalian metabolism. Early *Xenopus* embryos do not produce significant amounts of cytochrome P-450 so the exogenous system is necessary. Previous experimentation has shown that S-9 preparations do not contain enough cytochrome P-450 activity to be useful in FETAX. Aroclor 1254-induced microsomes do contain adequate activity for use as long as the protein content is not too high. Aroclor 1254 is used as a broad spectrum inducer and should be used in the majority of situations. Uninduced microsomes are used in those cases where Aroclor induction may repress some of the minor cytochromes. Isoniazid-induced microsomes are used for those test materials metabolized by cytochrome P-450j. The nature of the test material may suggest which inducing system to use.

Several compromises are required when MAS is employed. Sterile plastic Petri dishes are used to minimize bacterial contamination. These dishes hold only 8 ml instead of the 10 ml contained in the glass dishes. Antibiotics are also required to inhibit bacterial growth and these may interact with the test substance. Microsomal protein can slow growth and development at concentrations greater than 100 µg/ml. NADPH which is required for microsomal activity can also cause abnormal development and its concentration must be kept low. Despite these drawbacks, the MAS can improve the predictive accuracy of FETAX and provide repeatable and reliable data.

Each lot of microsomes is different. Thus, the cytochrome P-450 activity must be measured and a standard amount added to each dish. Controls are important in these experiments. It is important to perform a MAS-only (microsomes and generator system but no test material) negative control as well as a positive control for bioactivation. The bioactivation positive control is 4 mg/ml cyclophosphamide (Sigma Catalog # C 0768) with and without MAS. With MAS, the cyclophosphamide should kill 100% of the embryos in 96 hr while there should be no deaths without MAS. A final control is needed to demonstrate that the cytochrome P-450 system is responsible for the observed bioactivation. For this control, a small amount of dithionite (Sigma Catalog # S 1256) is added to dishes containing MAS and a concentration of test material that results in significant mortality or malformation upon bioactivation. After the mixture is gassed with carbon monoxide for three minutes, the cytochrome P-450 should be inactivated and the mortality and malformation rates should be as if no microsomes were present. The following references provide additional information on the use of MAS with FETAX.

Reagents Needed for Rat Liver

Microsome Preparation

Buffers

1. **0.05 M Tris-HCl** (Sigma Catalog # T 4128): Add 1.97 g of Tris-HCl to 250 ml of ASTM Type I water (ASTM standard D1193, Specification for Reagent Grade Water, Annual Book of ASTM Standards Vol. 11.01). Adjust pH to 7.5. Store at 4 °C.
2. **1.12% w/v KCl in 0.05 M Tris-HCl**: Add 3.94 g of Tris-HCl and 5.6 g of KCl to 500 ml of Type I water. Adjust pH to 7.5. Store at 4 °C.
3. **1.15% w/v KCl in 0.02 M Tris-HCl with 0.5% w/v bovine serum albumin (BSA; Sigma Catalog # A 9647)**: Add 1.57 g of Tris-HCl, 5.75 g KCl, and 2.5 g BSA to 500 ml of Type I water. Adjust pH to 7.5. Store at 4 °C.

Microsomal P-450 Preparation from Rat Liver

Animal Treatment

Male Sprague-Dawley rats (200-250 g) are used. For Aroclor 1254-induced microsomes, give an intraperitoneal injection of 500 mg/kg body weight. Allow five days for full induction. The Aroclor 1254 stock solution should be prepared in corn oil (500 mg/ml). For isoniazid induction use 0.1% w/v isoniazid (Sigma Catalog # I 3377) for ten consecutive days administered in the drinking water.

Preparation:

NOTE: Keep all buffers at 4 °C.

1. Kill the rat by cervical dislocation.
2. Begin perfusion of the liver via the hepatic portal vein with Buffer 2. Perfuse until well blanched (approximately 50 ml).
3. Excise liver and homogenize in seven volumes of Buffer 3 using a Potter-Elvehjem (P/E) homogenizer.
4. Centrifuge first at 900 x g for 10 min then increase speed to 9000 x g for 15 min.
5. Remove S-9 supernatant to another tube and centrifuge the S-9 supernatant at 105,000 x g for 1 hr.
6. Discard supernatant and resuspend pellet in Buffer 2.
7. Centrifuge again at 105,000 x g for an additional hr.

8. Resuspend microsomal pellet in 20-30 ml of Buffer 1 (depending on number of rats). Homogenize again with two to three strokes using a P/E or Dounce homogenizer.
9. Aliquot samples into 1.5 ml microcentrifuge tubes or cryovials, and snap freeze in liquid nitrogen. Measure protein (Bio-Rad kit; Bradford, M., Analytical Biochemistry 72: 248, 1976) and N-demethylase activity (see below) prior to use.

Metabolic Activation Generator System for FETAX

The following components can be added individually to the Petri dish or as a combined generator stock solution (see below). For routine work, use the combined generator stock.

1. Glucose-6-Phosphate - FW = 298.2 g/mole (Sigma Catalog # G 5137). 1 g is dissolved in 4 ml of FETAX solution yielding a 838.4 mM solution. 34 μ l of this solution in a total of volume of 8 ml per dish results in a final concentration of 3.6 mM.
2. Glucose-6-Phosphate Dehydrogenase (Sigma Catalog # G 7878). 250 U are dissolved in 2.5 ml FETAX solution. When 25 μ l are added to a total volume of 8 ml per dish the resulting concentration is 0.31 U/ml.
3. Nicotinamide Adenine Dinucleotide (NADP) - FW = 765.4 g/mole (Sigma Catalog # N 0505). 0.15 g is dissolved in 5 ml of FETAX solution. This results in a 39.2 mM solution. When 22 μ l is added to a total volume of 8 ml, the final concentration is 0.1 mM.
4. Reduced Nicotinamide Adenine Dinucleotide (NADPH) - FW = 743.4 g/mole (Sigma Catalog # N 1630). 5 mg is dissolved in 2.5 ml of FETAX solution resulting in a 2.7 mM solution. If 21 μ l is diluted to a total volume of 8 ml, the final concentration will be 7 μ M.
5. Combined Generator Stock Preparation (for 50 dishes). To make up the generator solution, add 1.85 g of glucose-6-phosphate, 132 mg NADP, 8.4 mg NADPH to 16.8 ml of FETAX. This will be enough to supply 50 dishes. Glucose-6-phosphate dehydrogenase is added separately. Add 77 μ l of the generator stock preparation to each dish. Store generator at -20 °C.

Antibiotics:

The number of units/mg varies with each lot of antibiotic. Make up a stock solution of 10,000 U/ml penicillin G (Sigma Catalog # PEN-NA) and 10,000 U/ml streptomycin sulfate (Sigma Catalog # S 6501) in FETAX solution. The dilution factor is 100 and 80 μ l should be added to each dish of 8 ml total to give a final concentration of 100 U/ml of each antibiotic. Store at 4 °C.

N-Demethylase Determination by a Modified Procedure from Nash (1955) and Lucier et al. (1971).

Background:

Aminopyrine is used as a substrate when determining the P-450 activity of Aroclor 1254-induced microsomes while N-dimethylnitrosamine is used for isoniazid-induced microsomes. Aminopyrine is converted into aminoantipyrine and formaldehyde. Dimethylnitrosamine is metabolized into monomethylnitrosamine and formaldehyde. By using a colorimetric assay using acetyl acetone in the presence of acetic acid and ammonium ion analyzed at 412 nm, the amount of formaldehyde produced can be determined.

Reagents and Stock Solutions for Nash Assay

1. **Nash Reagent** - 22.5 g of ammonium acetate (Sigma Catalog # A 7262; FW 77.08; 6 M final concentration), 0.3 ml acetylacetone (Sigma Catalog # A 3511; MW 100.1; 60 mM final concentration) and 0.75 ml acetic acid (Sigma Catalog # A 6283; FW 60.05; 0.15 M final concentration) in 50 ml distilled water, pH to 6.7. Store at 4 °C.
2. **840 mM Glucose-6-Phosphate** (FW 298.2) - 250.5 mg in 1 ml of Buffer 1. Store at -20 °C.
3. **2.5 mM NADPH** (FW 833.4) - 2.08 mg in 1 ml of Buffer 1. Store at -20 °C.
4. **235 mM MgCl₂** (FW 203.31) - 4.78 g in 100 ml of Buffer 1. Store at 4 °C.
- 5a. For Aroclor-induced microsomes:
10 mM aminopyrine (Sigma Catalog # D 8015)(FW 231.3) - 23.13 mg in 10 ml of Buffer 1. Store at 4 °C.
- 5b. For Isoniazid-induced microsomes:
10 mM dimethylnitrosamine (DMNA) (Sigma Catalog # N 3632)(FW 74.08) -
 1. To a 1 g Sigma Isopak add 50 ml of Buffer 1 (270 mM final concentration).
 2. 0.371 ml of 270 mM DMNA is then added to 10 ml of Buffer 1 (10 mM final concentration). Store at 4 °C.
6. 10% w/v trichloroacetic acid (TCA)(Sigma Catalog # T 6399) - 10 mg in 100 ml of Buffer 1. Store at room temperature.

Procedure:

1. Prepare microsomes as previously described.
2. Each 3 ml sample contains the following:
 - a. 5 mM Glucose-6-phosphate - 18 μ l of 840 mM (Glucose-6-Phosphate)
 - b. 0.4 mM NADPH - 480 μ l of 2.5 mM (NADPH)
 - c. 20 mM $MgCl_2$ - 255 μ l of 235 mM ($MgCl_2$)
 - d1. For Aroclor-induced microsomes:
8 mM Aminopyrine - 540 μ l of 10 mM (Aminopyrine)
 - d2. For Isoniazid-induced microsomes:
1.8 mM Dimethylnitrosamine - 540 μ l of 10 mM (DMNA)
 - e. 2 Units Glucose-6-Phosphate dehydrogenase.
Use single unit assay vials (Sigma Catalog # G 1256) and dilute the enzyme with 0.5 ml per vial of Buffer 1. Add 1 ml total to the 3 ml sample.
 - f. 100 μ l of microsomal sample.
 - g. Adjust volume to 3.0 ml with Buffer 1. (For the additions above, 607 μ l should be added.).
3. Incubate mixture at 37 °C for 10 min.
4. Remove 1 ml of the incubation mixture and add 1.5 ml of 10% w/v trichloroacetic acid (TCA) to this aliquot. (The sample can be split into two replicates at this time.)
5. Centrifuge sample at 12,000 x g for 20 min., room temperature.
6. Remove 2 ml of the clear supernatant and place in a capped tube.
7. Add 1 ml of the Nash reagent and incubate in the water bath for 10 min at 60 °C.
8. Measure Absorbance at 412 nm. Formaldehyde has an extinction coefficient of 7950/(cm x M).

Absorbance calculation:

$A = (\text{absorbance of sample} - \text{absorbance of blank})$
 $3.75 = \text{dilution factor.}$
 $0.00795/(\text{cm} \times \mu\text{M}) = \text{extinction coefficient}$
10 min is the length of time of the assay.
0.1 is the ml of microsomal sample used in the assay.

$A \times (3.75/0.00795) \times 1/10 \text{ min} \times 1/0.1 \text{ ml microsomal sample} = \mu\text{M formaldehyde per min per ml.}$
Final units are $\mu\text{M formaldehyde per min per ml of microsomes.}$

Dividing by the protein concentration yields the $\mu\text{M formaldehyde per minute per mg microsomal protein.}$
1 $\mu\text{M formaldehyde/min}$ is an APD unit. We generally add 0.4 APD Units per ml. The activity must be ≥ 6.67 APD Units per mg protein.

Standard Curve (If you do not wish to use the extinction coefficient you may use the standard curve)

- 0.1 mg/ml formaldehyde stock
- 0.5 $\mu\text{g/ml}$ - 0.015 ml stock and 1.985 ml buffer 1
- 1.0 $\mu\text{g/ml}$ - 0.03 ml stock and 1.97 ml buffer 1
- 5.0 $\mu\text{g/ml}$ - 0.15 ml stock and 1.85 ml buffer 1
- 10.0 $\mu\text{g/ml}$ - 0.30 ml stock and 1.7 ml buffer 1
- 50.0 $\mu\text{g/ml}$ - 1.5 ml stock and 0.5 ml buffer 1

Calculations:

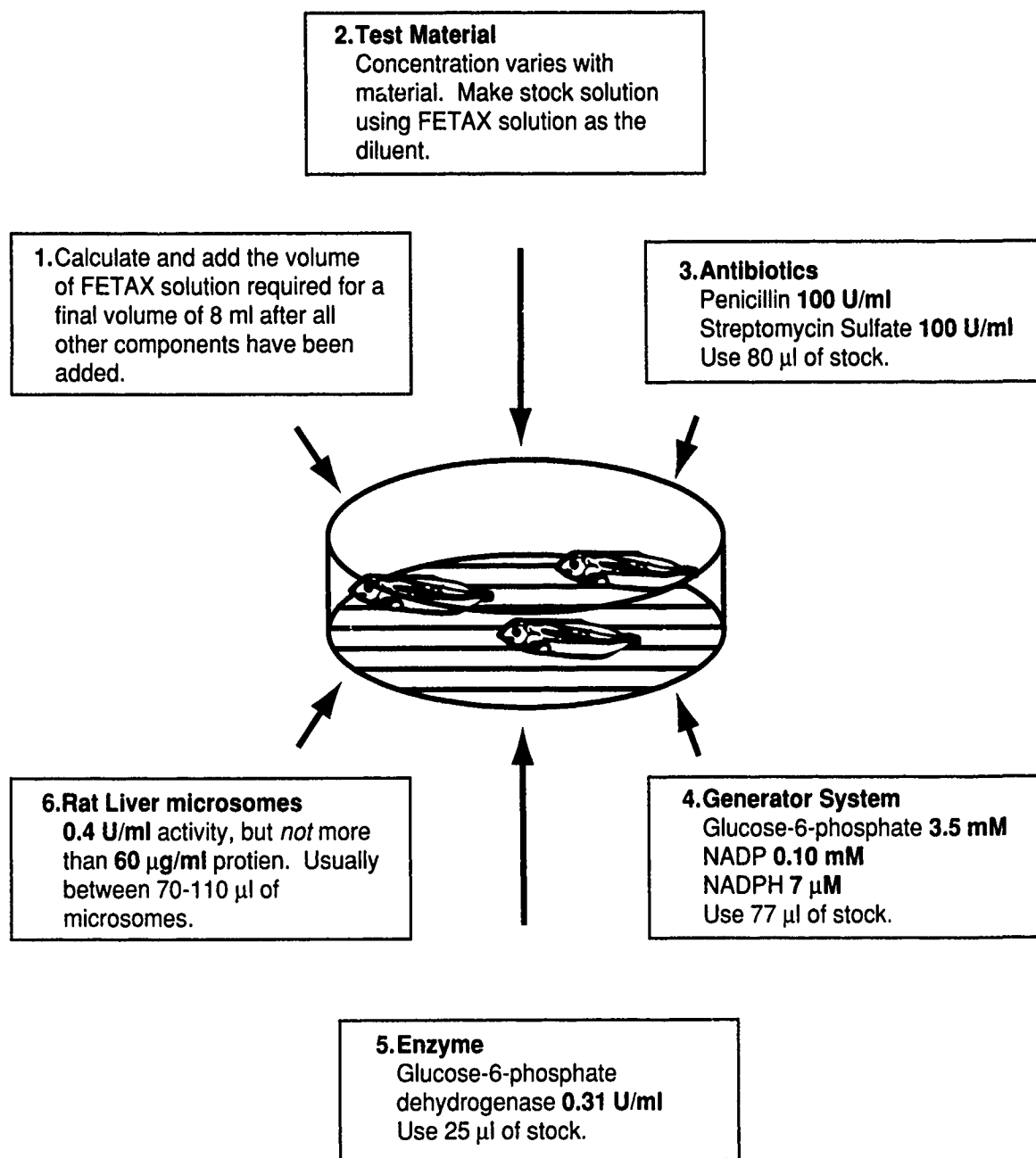
Regression analysis is used to determine the concentration of formaldehyde. In order to determine the formaldehyde concentration in 3 ml of incubation media multiply the regression analysis value by 3.75. This is the amount of formaldehyde created by 0.1 ml of protein. Divide this result by 10 min and the protein concentration to obtain the $\mu\text{g of formaldehyde/minute/mg protein.}$

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Summary of Metabolic Activation System

Components added to Each Petri Dish



A sterile 60x15 mm plastic Petri dish is used. There are a total of 8 mls in each dish. The diluent is FETAX solution and the concentrations in **boldface** are the final concentrations for each component. The number on each box indicates the order of addition.

Other Procedures

Staining *Xenopus laevis* Embryos.

At times, it might be desirable to observe surface structures of embryos. This is especially true in training situations when a new technician is attempting to stage 96-hr larvae or to view facial malformations. The following technique uses crystal violet and acid decolorization to stain selectively certain denser structures such as the brain, cartilage, and the hind limb bud. It also highlights surface structures such as the external nares, tail fin, and mouth. Once observed with the stain, these structures are much more easily identified in the unstained organism (See Chapter 8, Figure 3).

Modified Gram's Stain for 96-hr Embryos

Note: Embryos should have been in 3% formalin for at least 24 hr. Freshly killed and fixed embryos will not stain the same way.

CAUTION: Do not allow embryos to dry out during solution changes.

- 1) Remove embryos from formalin.
- 2) Wash embryos twice with FETAX solution.
- 3) Place embryos in a 60 mm Petri dish with 10 ml FETAX solution.
- 4) Add 3 drops Hucker's crystal violet.
- 5) Wait 30 seconds.
- 6) Drain Hucker's crystal violet/FETAX solution from embryos.
- 7) Rinse with FETAX solution until solution remains clear.
- 8) Drain completely.
- 9) Use just enough dilute acid alcohol to cover embryos.
- 10) Wait 2 to 10 seconds depending on decolorization desired.
- 11) Add 10 ml of FETAX solution while swirling to further dilute the acid alcohol and arrest decolorization.
- 12) Immediately drain completely.
- 13) Rinse twice with FETAX solution.
- 14) Replace FETAX solution with 3% formalin.

Note: The color will change slightly after the addition of formalin, and will eventually fade away completely, but should be visible for several weeks.

Hucker's Crystal Violet:

Mix equal volumes of Solution A and Solution B:

Solution A:

2 g Crystal Violet (90% dye content)

20 ml 95% Ethyl Alcohol

Solution B:

0.8 g Ammonium Oxalate

80 ml Distilled Water

Dilute Acid Alcohol:

5% v/v of Acid Alcohol in FETAX solution (5 ml Acid Alcohol + 95 ml FETAX solution = 100 ml of Dilute Acid Alcohol)

Acid Alcohol:

3% v/v of concentrated HCl in 95% Ethyl Alcohol I + 95 ml FETAX solution = 100 ml of Acid Alcohol)

Observation of Live *Xenopus laevis* Embryos.

MS-222 (Sigma Catalog # A 5040) can be successfully used to immobilize and observe live embryos for staging, heart abnormalities, and other defects by the addition of approximately 0.02 mg of MS-222 per 10 ml of FETAX solution. The powder should be added directly to a 60 mm Petri dish containing embryos in 10 ml of FETAX solution. Higher concentrations will kill the embryos. After 10-15 seconds, the embryos will be motionless and can be rotated onto their backs for ventral examination, or rotated onto their sides for staging by the observation of the hind limb bud. After observation, the embryos can be revived by placement in fresh FETAX solution or they can be formalin-fixed.

Preparation of *Xenopus laevis* 96-hr Embryos for Scanning Electron Microscopy(SEM).

The following procedure is a standard method for SEM preparation of biological specimens.

1. Fix 96-hr embryo with 1.6% gluteraldehyde for two hours.
2. Wash with phosphate buffer three times.
3. Post-fix tissue in 2% OsO₄ for two hours.
4. Dehydrate in ethanol, 6 washes from 50% to 100% ethanol.
5. Dry using Critical Point Dryer (CPD)(place on stub).
6. Put in desiccator overnight.
7. Au-Pd sputter coat just before viewing.
8. Store in dust-free, dry area, such as a desiccator.

Step 3 may not be necessary, but helps to harden the tissue. Special attention should be paid to the dehydration and CPD steps. Any water in the sample during CPD will shrivel the embryos and make them unusable. However, shriveled embryos will usually show the cilia that cover the embryo, particularly in the nasal area, in good condition.

An alternative to CPD is freeze drying. The fixation wash is as above except in step 4, transfer the tissue into distilled water, wash three times and then freeze in liquid nitrogen. Place in a freeze dryer and when dry, place on stub and continue at step 6 above. Freeze drying does not shrivel the embryos as much as CPD. However mechanical damage may be a problem. The cilia are not as visible as with CPD.

The method of preference is CPD, which seems to give the best representation of the true embryo appearance as with CPD.

Reference:

Pelczar, M.J., Laboratory Exercises in Microbiology, 1965, 339.

Acknowledgments

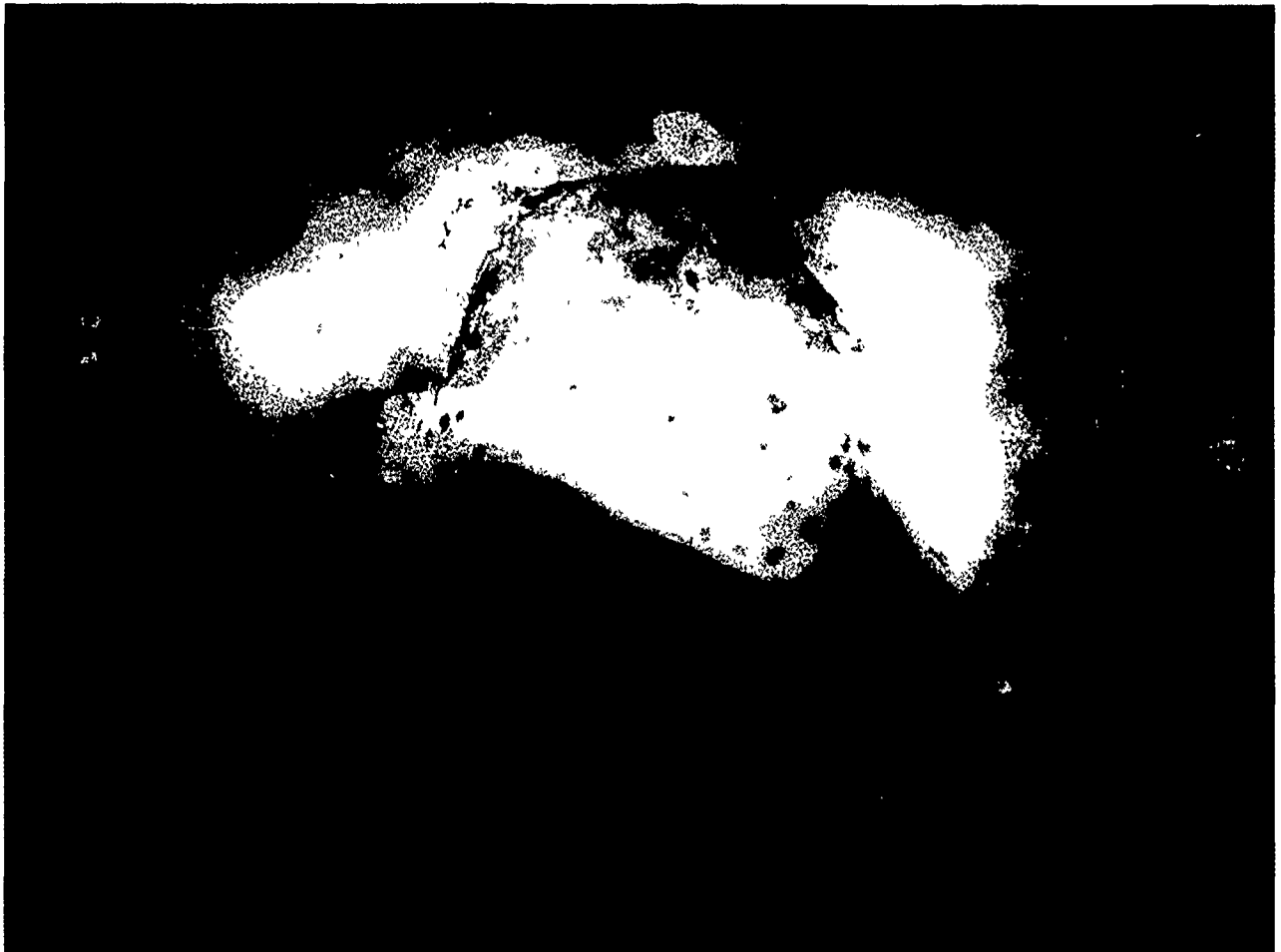
The authors gratefully acknowledge the tremendous help that Ms. Mendi Hull has provided on this project. She produced many of the photographs, typed most of the text, and edited the Atlas numerous times. Ms. Donna DeYoung contributed several excellent diagrams which

supplemented the photographs. Other contributors were Dr. Doug Dawson, Dr. Doug Fort, Mr. James Rayburn and Ms. P.K. Work. These individuals contributed either photographs or constructive advice. We appreciate their help in this undertaking.

ANYTHING IS POSSIBLE!

This two-headed embryo is probably the most unusual embryo produced in FETAX testing to date. It is possible that two embryonic axes were established very early in development by the action of the test material. This action could have been similar to early

experimental embryology experiments where a second dorsal blastopore lip was grafted 180 degrees apart from the normal dorsal lip. Essentially, two embryos develop in opposite directions (photo by Hull).



Evaluation of the Developmental Toxicity of Five Compounds with the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) and a Metabolic Activation System

Douglas J. Fort, Brenda L. James and John A. Bantle†

Department of Zoology, 430 Life Sciences West, Oklahoma State University, Stillwater, OK 74078, USA

Key words: FETAX; developmental toxicity; 2-acetyl aminofluorene; rifampicin; benzo[a]pyrene; zinc; cytochalasin D.

The potential teratogenic hazard of five compounds was evaluated using the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) and a metabolic activation system. Embryos of the South African clawed frog, *Xenopus laevis*, were exposed to (i) three compounds suspected to be proteratogenic in mammalian test systems—[2-acetylaminofluorene (2-AAF), rifampicin (RA) and benzo[a]pyrene (BP)] for 96 h; (ii) one compound unaffected by mixed-functional oxidase (MFO) metabolism—ZnSO₄; (iii) one compound thought to be inactivated by cytochrome P-450—cytochalasin D (CD). Two separate static renewal tests were conducted with and without the presence of an exogenous metabolic activation system (MAS). The metabolic activation system consisted of Aroclor 1254-induced rat liver microsomes. The teratogenic potential of each compound and the effects of metabolic activation were based on teratogenic indices [TI=96 h LC₅₀/96 h EC₅₀ (malformation)], types and severity of malformation, and effects on embryo growth. Metabolic activation increased the potential teratogenic hazard of 2-AAF, RA and BP by TI factors of 1.3, 2.8 and 6.8, respectively. The teratogenic potential of ZnSO₄ was virtually unaffected by the MAS. The MAS significantly reduced the teratogenic potential of CD by a TI factor of 2.7. These results demonstrate the utility and importance of a MAS for *in vitro* developmental toxicity screens such as FETAX. Consistent use of a MAS with FETAX should reduce the number of potential false-positive and false-negative test results.

INTRODUCTION

Increasing demands for developmental toxicity screening necessitate the development, validation and use of alternative screening systems to the traditional mammalian test systems.¹ *In vitro* teratogenesis screening systems may provide a rapid, cost-effective method of determining compounds and complex mixtures that may be potential teratogenic hazards. A fundamental problem shared by many *in vitro* developmental toxicity screening systems is the inability to metabolize xenobiotics.² This presents a major obstacle in the detection of proteratogens, as well as teratogens inactivated by the mixed-functional oxidase (MFO) system.

The Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) is a 96-h static renewal bioassay designed to screen potential developmental toxicants.³ Since *Xenopus* embryos lack many metabolic enzyme systems through the first 96 h of development, an exogenous metabolic activation system (MAS) was developed for FETAX using Aroclor 1254-induced rat liver microsomes and the proteratogen cyclophosphamide (CP).⁴ Recently, the developmental toxicity of nicotine and a primary metabolite cotinine on *Xenopus* embryos was evaluated using this MAS.⁵ Bioactivation greatly reduced the teratogenicity and growth inhibiting effects of nicotine. This report describes the evaluation of the

developmental toxicity of three compounds suspected to be proteratogenic—2-acetylaminofluorene (2-AAF), rifampicin (RA) and benzo[a]pyrene (BP)—one compound unaffected by MFO metabolism—ZnSO₄—and one compound believed to be metabolically inactivated by the MFO system—cytochalasin D (CD). The results demonstrate the importance of a successful MAS for *in vitro* teratogenesis screening systems such as FETAX, as well as the utility of these systems in evaluating the role that MFO metabolism plays in teratogenesis.

EXPERIMENTAL

Chemicals and biochemicals

All chemicals used in the rat liver microsome preparation, test compounds and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Aroclor 1254 was obtained from Monsanto Corporation (St. Louis, MO).

Microsome preparation

Aroclor 1254-induced rat liver microsomes were prepared as described previously.⁴ Adult male Sprague-Dawley rats (200–300 g) were injected with 500 mg kg⁻¹ Aroclor 1254 in corn oil for five days prior to microsome preparation.⁶ Following cervical dislo-

† Author to whom correspondence should be addressed.

cation, the liver was exposed and perfused with 50 ml of 0.02 M Tris-HCl buffer containing 1.15% KCl (pH 7.5). The liver was homogenized in 1.15% KCl-0.02 M Tris-HCl buffer containing 0.5% bovine serum albumin (pH 7.5). The homogenate was then centrifuged successively at 600, 9000 and 102 000 g ave, resuspended and pelleted again. The final washed microsomal pellet was suspended in 0.05 M Tris-HCl (pH 7.5), put into 1.5-ml aliquots and immediately frozen in liquid nitrogen.⁷ Protein content was determined by the method of Bradford.⁸ Cytochrome P-450 (P-450) activity was inferred by the measurement of formaldehyde generated from the *N*-demethylation of aminopyrine (APD)⁹ under standard assay conditions established previously.¹⁰ Several aliquots of microsomes were chemically reduced with dithionite and pretreated with carbon monoxide (CO-MAS) to selectively inactivate P-450 activity.¹¹

Animal care and breeding

Xenopus adult care, breeding and embryo collection were performed according to Courchesne and Bantle.¹²

Assay procedure

Initial tests were conducted to determine the maximum amount of DMSO that could be used as diluent and the effect that it had on P-450 activity. For unactivated tests, groups of 20 embryos were placed in 60-mm covered plastic Petri dishes with varying concentrations of the appropriate test compound. 2-AAF, RA, BP and CD stock solutions were prepared by dissolving in 1% (v/v) DMSO diluted in FETAX solution—a reconstituted water medium appropriate for the culturing of *Xenopus* embryos.¹³ ZnSO₄ was dissolved in appropriate volumes of FETAX solution. For each compound, 8–13 concentrations were tested with replicates. Four separate dishes of 20 embryos were exposed to FETAX solution (designated FETAX solution controls). Forty embryos, 20 per dish, were exposed to 1% DMSO in FETAX solution and served as solvent controls. Each treatment contained a total of 8 ml of solution.

Stock Zn concentrations were determined by atomic absorption analysis. To facilitate the comparison of tests within this study and previous work with Zn,¹⁴ the concentration of each test dilution was normalized to 100 $\mu\text{g ml}^{-1}$ hardness (as CaCO₃).^{15,16}

The metabolically activated tests were also conducted in duplicate with 20 embryos per dish. Stock solutions were prepared as described for the unactivated tests. Each activated treatment received 0.04 U APD activity (units expressed as μM formaldehyde min^{-1}), an NADPH generating system and 100 U ml^{-1} penicillin–100 $\mu\text{g ml}^{-1}$ streptomycin. The NADPH generating system consisted of 3.5 mM glucose-6-phosphate, 0.31 IU ml^{-1} glucose-6-phosphate dehydrogenase, 0.1 mM NADP and 7.0 μM NADPH. Each dish received no more than 0.06 mg ml^{-1} microsomal protein.⁴ Controls for FETAX solution, DMSO, MAS, DMSO + MAS, CP (FETAX reference proteratogen),⁵ CO-MAS + toxicant (negative control) and unactivated toxicant were also run with each test.

For each compound, one range-finder and two separate definitive tests were conducted with and without the MAS. The pH of all stock solutions was maintained between 7.0 and 7.5. Embryos were cultured at 23°C throughout the test. All solutions were removed every 24 h of the 4-day test and fresh solutions were added. Dead embryos were removed at this time. After 96 h of exposure, surviving embryos were fixed in 0.7% formalin (pH 7.0). The number of live malformed embryos and the stage of development¹⁷ were determined using a dissecting microscope.

Data analysis

Litchfield–Wilcoxon probit analysis was used to ascertain the 96-h LC₅₀ (the median lethal concentration) and the 96-h EC₅₀ (the concentration inducing gross terata in 50% of the surviving embryos).¹⁸ The 95% confidence limits were calculated as well. A Teratogenic Index (TI), which has proved useful in assessing teratogenic hazards,^{3–5,13,19} was determined by dividing the 96-h LC₅₀ by the 96-h EC₅₀ (malformation). Head–tail length (growth) was measured using a Radio Shack digitizer and a model 16 microcomputer.

RESULTS

Control experiments

Preliminary tests indicated that upon exposure to 1% (v/v) DMSO, microsomal APD activity was 0.05 U mg^{-1} protein \pm 0.01 ($n=6$), which was not significantly different from FETAX solution controls, 0.05 U mg^{-1} protein \pm 0.03 ($n=6$) (grouped *t*-test, $P=0.05$). The lowest observable effect concentration (LOEC) (Dunnett's test, $P=0.05$) and the minimum concentration to inhibit growth (MCIG) (grouped *t*-test, $P<0.05$) for the embryos exposed to DMSO for 96 h were 1.4 and 1.2% (v/v), respectively.

In all tests conducted, FETAX solution control embryo mortality and malformation rates were $<6\%$. Mortality and malformation rates in the MAS and DMSO controls were below 6 and 7%, respectively. Mortality and malformation rates in control embryos exposed to the MAS and DMSO simultaneously were 15% or less. At least 90% of the embryos exposed to 4.0 mg ml^{-1} activated CP died before 96 h. Survivors were severely malformed. Control embryos exposed to 4.0 mg ml^{-1} unactivated CP exhibited malformation and mortality rates less than or equal to 15%.

2-Acetylaminofluorene

Results from tests with 2-AAF are presented in Table 1. The mean unactivated 96-h LC₅₀ was 88.5 $\mu\text{g ml}^{-1}$. The mean 96-h EC₅₀ (malformation) was 7.2 $\mu\text{g ml}^{-1}$, yielding a TI of 12.4. Metabolic activation reduced the 96-h LC₅₀ at least 1.9-fold, to ca. 42.5 $\mu\text{g ml}^{-1}$. Activation reduced the 96-h EC₅₀ (malformation) to approximately 2.6 $\mu\text{g ml}^{-1}$ (2.7-fold). The average TI value was 16.7. Malformations induced by unactivated 2-AAF included improper gut coiling and pericardial

Table 1. Effect of 2-acetylaminofluorene on *Xenopus* embryo development

| Treatment | Unactivated | | Activated | |
|---|-------------|-------------|-------------|-------------|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| FETAX solution control ^a | | | | |
| Mortality | 5.0 | 0 | 1.3 | 0 |
| Malformation | 3.5 | 2.5 | 2.5 | 5.0 |
| MAS ^b control ^a | | | | |
| Mortality | - | - | 5.0 | 0 |
| Malformation | - | - | 3.4 | 5.0 |
| 1% (v/v) DMSO control ^a | | | | |
| Mortality | 2.5 | 0 | 0 | 0 |
| Malformation | 5.1 | 5.0 | 5.0 | 5.0 |
| MAS + 1% (v/v) DMSO ^a | | | | |
| Mortality | - | - | 7.5 | 0 |
| Malformation | - | - | 15.0 | 12.5 |
| 4.0 mg ml ⁻¹ CP ^{a,c} | | | | |
| Mortality | 0 | 0 | 100.0 | 100.0 |
| Malformation | 20.0 | 10.0 | - | - |
| CO-MAS ^d + 75 µg ml ⁻¹ 2-AAF ^{a,e} | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 100.0 | 85.0 | 100.0 | 100.0 |
| LC ₅₀ ^f (µg ml ⁻¹) | 87.0 | 90.0 | 45.0 | 40.0 |
| | (84.0-89.0) | (88.0-93.0) | (26.0-78.0) | (32.0-58.0) |
| EC ₅₀ ^f (µg ml ⁻¹) | 6.9 | 7.4 | 2.6 | 2.5 |
| | (6.5-7.3) | (7.0-8.0) | (0.4-9.0) | (2.0-3.0) |
| TI ^g | 12.6 | 12.2 | 17.3 | 16.0 |

^a Data presented as percent effect.

^b Metabolic activation system.

^c Cyclophosphamide.

^d Carbon monoxide-inactivated MAS.

^e Unactivated column represents data for embryos exposed to 75 µg ml⁻¹ 2-AAF alone.

^f Determined by Litchfield-Wilcoxon probit analysis with 95% confidence interval in parentheses.

^g 96-h LC₅₀/96-h EC₅₀ (malformation).

edema at concentrations >6 µg ml⁻¹. At concentrations >10 µg ml⁻¹, severe gut malformations, ophthalmic edema and craniofacial abnormalities were also observed. Gut miscoiling, edema of the pericardium, ophthalmic region and dorsal fin, and severe microphthalmia were elicited by activated 2-AAF at concentrations of >1.0 µg ml⁻¹. Concentrations of >10 µg ml⁻¹ bioactivated 2-AAF produced severe skeletal kinking, malformations of the mouth and microencephaly. The effects of unactivated and bioactivated 2-AAF on embryo growth is illustrated in Fig. 1. Exposure to metabolically activated 2-AAF had a greater growth inhibiting effect than did exposure to unactivated 2-AAF. Growth reduction as the result of 2-AAF bioactivation was also greater than the additive growth inhibition caused by the MAS + DMSO and unactivated 2-AAF treatments. Embryos exposed to CO-MAS and 85 µg ml⁻¹ 2-AAF were 75.5% of FETAX solution control growth.

Rifampicin

Unactivated RA was not developmentally toxic at the limit of solubility in 1% (v/v) DMSO, thus the 96-h LC₅₀ and EC₅₀ (malformation) values are expressed as >2.0 mg ml⁻¹ (Table 2). Bioactivation decreased the 96-h LC₅₀ to ca. 1.4 mg ml⁻¹. The mean 96-h EC₅₀ (malformation) for bioactivated rifampicin was 0.5 mg ml⁻¹ and the TI was 2.8. Activated RA concentrations of >50 µg ml⁻¹ caused miscoiling of the gut, craniofacial deformities and microphthalmia. Severe microencephaly, ophthalmic and pericardial edema, and skeletal kinking were produced in concentrations of >1.0 mg ml⁻¹. The effect of metabolic activation of RA on embryo growth is presented in Fig. 2. Activated RA concentrations of >1 mg ml⁻¹ caused slight growth inhibition. However, the reductions may have been caused by the sum of the actions of the MAS + DMSO and unactivated RA treatments. Unactivated RA

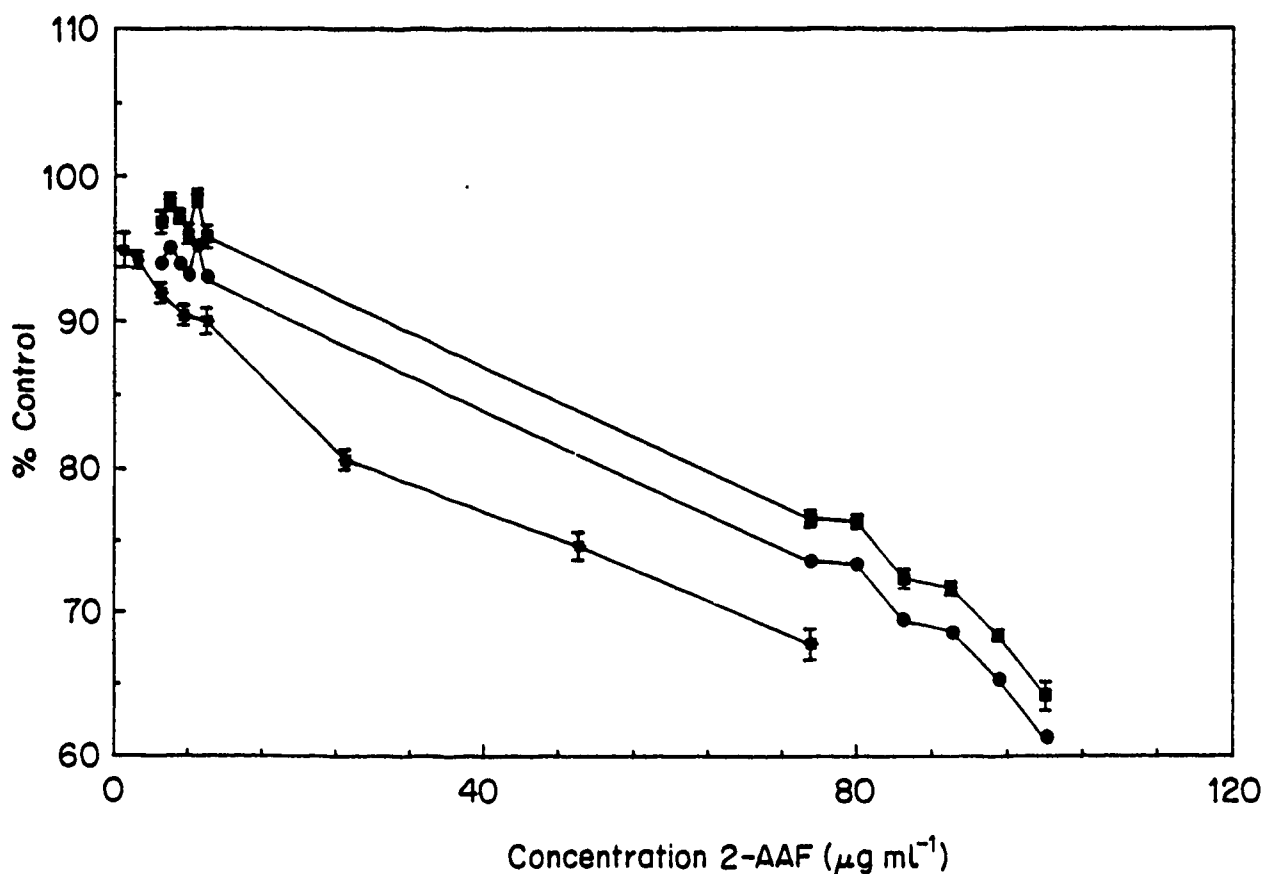


Figure 1. Representative growth curves from final definitive experiments, presented as percent of mean FETAX solution control with SE, for *Xenopus* embryos exposed to unactivated (\blacksquare) and bioactivated (\star) 2-AAF for 96 h; (\bullet) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated 2-AAF treatments.

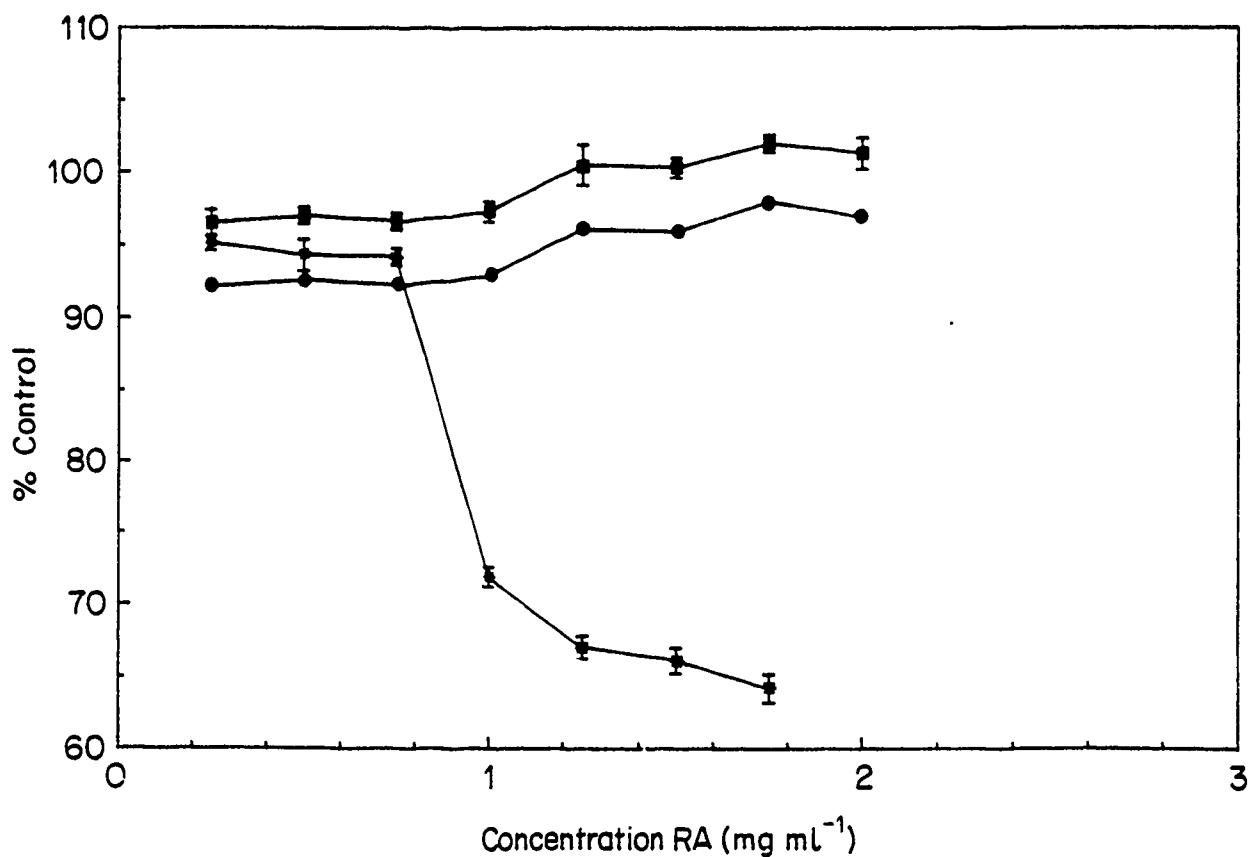


Figure 2. Representative growth curves from final definitive experiments, presented as percent of mean FETAX solution control with SE, for *Xenopus* embryos exposed to unactivated (\blacksquare) and bioactivated (\star) RA; (\bullet) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated RA treatments.

Table 2. Effect of rifampicin on *Xenopus* embryo development

| Treatment | Unactivated | | Activated | |
|--|-------------------|---------|---------------------|---------------------|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| FETAX solution control ^a | | | | |
| Mortality | 1.9 | 1.9 | 2.5 | 0 |
| Malformation | 3.4 | 5.1 | 5.1 | 0 |
| MAS ^b control ^a | | | | |
| Mortality | - | - | 2.5 | 0 |
| Malformation | - | - | 5.1 | 5.0 |
| 1% (v/v) DMSO control ^a | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 5.0 | 2.5 | 5.0 | 0 |
| MAS + 1% (v/v) DMSO ^a | | | | |
| Mortality | - | - | 2.5 | 0 |
| Malformation | - | - | 10.3 | 5.0 |
| 4.0 mg ml ⁻¹ CP ^{a,c} | | | | |
| Mortality | 0 | 0 | 100.0 | 100.0 |
| Malformation | 5.0 | 5.0 | - | - |
| CO-MAS ^d + 2.0 mg ml ⁻¹ RA ^{a,e} | | | | |
| Mortality | 0 | 13.3 | 0 | 0 |
| Malformation | 5.0 | 15.4 | 15.4 | 10.0 |
| LC ₅₀ ^f (mg ml ⁻¹) | >2.0 ^g | >2.0 | 1.40 (1.24-1.56) | 1.35 (1.26-1.46) |
| EC ₅₀ ^f (mg ml ⁻¹) | >2.0 | >2.0 | 0.53 (0.45-0.63) | 0.47 (0.39-0.55) |
| TI ^h | - | - | 2.6 | 2.9 |
| ^a Data presented as percent effect. ^b Metabolic activation system. ^c Cyclophosphamide. ^d Carbon monoxide-inactivated MAS. ^e Unactivated column represents data for embryos exposed to 2.0 mg ml ⁻¹ RA alone. ^f Determined by Litchfield-Wilcoxon probit analysis with 95% confidence interval in parentheses. ^g Limit of solubility in 1% (v/v) DMSO. ^h 96 h LC ₅₀ /96 h EC ₅₀ (malformation). | | | | |

concentrations of >1.5 mg ml⁻¹ caused increased embryo growth compared to FETAX solution controls, whereas activated concentration of >1.0 mg ml⁻¹ caused marked growth reduction. Embryonic growth in the FETAX solution controls and the CO-MAS + 2.0 mg ml⁻¹ RA treatment was nearly identical.

Benzo[a]pyrene

Results from tests with BP are shown in Table 3. The 96-h LC₅₀ for both unactivated and metabolically activated BP is expressed as being >10 µg ml⁻¹, since the median lethal concentration exceeded the limit of solubility in 1% (v/v) DMSO. The 96-h EC₅₀ (malformation) for unactivated BP was ca. 11 µg ml⁻¹. Bioactivation reduced the 96-h EC₅₀ (malformation) by ca. 6.7-fold to 1.7 µg ml⁻¹. The TI increased at least 5.6-fold upon activation. At concentrations of >2.5 µg ml⁻¹, unactivated BP elicited moderate gut malformations, while concentrations of >7.5 µg ml⁻¹ produced severe gut abnormalities and malformations of the mouth. Bioactivation induced ophthalmic edema, severe gut miscoiling and mouth malformation at concentrations of >0.5 µg ml⁻¹. In addition, micro-

encephaly, severe eye malformations and skeletal kinking were observed at >5 µg ml⁻¹ activated BP. The effect of bioactivated BP on embryo growth is shown in Fig. 3. Activated BP concentrations greater than ca. 5 µg ml⁻¹ caused greater growth reduction than could be explained by the additive effect of the MAS + DMSO and unactivated BP treatments. Embryos exposed to 10 µg ml⁻¹ BP and CO-MAS were 87% of FETAX solution control growth.

ZnSO₄

In tests without the MAS, the average normalized 96-h LC₅₀ was 34.4 µg ml⁻¹ (Table 4). The 96-h EC₅₀ (malformation) was ca. 2.7 µg ml⁻¹ in tests without the MAS. In tests with the MAS, the mean 96-h LC₅₀ was 36.7 µg ml⁻¹. The activated LC₅₀ value (standardized per microgram of microsomal protein) was ca. 1.6 µg ZnSO₄ µg⁻¹ protein. With the MAS, the 96-h EC₅₀ (malformation) was ca. 2.9 µg ml⁻¹. The TI values for unactivated ZnSO₄ and ZnSO₄-MAS experiments were 13.3 and 12.7, respectively. Embryos exposed to ZnSO₄ alone at concentrations of >1.5 µg ml⁻¹ caused mild gut malformations and pericardial

Table 3. Effect of benzo(a)pyrene on *Xenopus* embryo development

| Treatment | Unactivated | | Activated | |
|--|--------------------|------------|-----------|-----------|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| FETAX solution control ^a | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 0 | 3.4 | 0 | 3.4 |
| MAS ^b control ^a | | | | |
| Mortality | - | - | 0 | 3.4 |
| Malformation | - | - | 2.5 | 3.4 |
| 1% (v/v) DMSO control ^a | | | | |
| Mortality | 2.5 | 0 | 2.5 | 0 |
| Malformation | 5.0 | 0 | 3.4 | 0 |
| MAS + 1% (v/v) DMSO ^a | | | | |
| Mortality | - | - | 0 | 0 |
| Malformation | - | - | 15.0 | 3.4 |
| 4.0 mg ml ⁻¹ CP ^{a,c} | | | | |
| Mortality | 0 | 0 | 100.0 | 100.0 |
| Malformation | 15.0 | 15.0 | - | - |
| CO-MAS ^d + 10 µg ml ⁻¹ BP ^{a,e} | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 0 | 0 | 40.0 | 33.3 |
| LC ₅₀ ^f (µg ml ⁻¹) | >10.0 ^g | >10.0 | >10.0 | >10.0 |
| EC ₅₀ ^f (µg ml ⁻¹) | 12.0 | 10.0 | 1.5 | 1.8 |
| | (6.0-20.0) | (8.0-12.0) | (1.0-2.4) | (1.2-3.0) |
| TI ^h | >0.8 | >1.0 | >6.7 | >5.6 |

^a Data presented as percent effect.

^b Metabolic activation system.

^c Cyclophosphamide.

^d Carbon monoxide-inactivated MAS.

^e Unactivated column represents data for embryos exposed to 10 µg ml⁻¹ BP alone.

^f Determined by Litchfield-Wilcoxon probit analysis with 95% confidence interval in parentheses.

^g Limit of solubility in 1% (v/v) DMSO.

^h 96-h LC₅₀/96-h EC₅₀ (malformation).

edema. Above 4.0 µg ml⁻¹, ZnSO₄ induced severe edema of the pericardium and eye, gut miscoiling, and head and mouth malformations. Higher concentrations also caused severe skeletal kinking, microphthalmia, microencephaly and craniofacial malformations. ZnSO₄-MAS exposure produced similar malformations to those induced by the unactivated treatments. The severity of the gut miscoiling and edema was slightly greater at the lower concentrations (<4.0 µg ml⁻¹) when cultured with the MAS. At higher concentrations (>5.0 µg ml⁻¹), skeletal kinking was observed less and was generally less severe than that produced in the unactivated treatments. The effect of ZnSO₄ on embryo growth is shown in Fig. 4. Addition of the MAS had little effect on the embryo growth inhibiting potential of ZnSO₄. Embryos exposed to 85 µg ml⁻¹ ZnSO₄ (30.5-36.3 µg ml⁻¹ normalized) and the CO-MAS were 81.7% of FETAX solution control growth.

Cytochalasin D

Table 5 shows the results of tests conducted with CD. The mean 96-h LC₅₀ for unactivated CD experiments

was 450 ng ml⁻¹. The inactivated 96-h EC₅₀ (malformation) was ca. 100 ng ml⁻¹. In tests conducted with the MAS, the 96-h LC₅₀ averaged 800 ng ml⁻¹. The bioinactivated 96-h EC₅₀ (malformation) was ca. 600 ng ml⁻¹. The TI for unactivated CD was ca. 3.9 whereas the TI for bioinactivated CD was ca. 1.5. Unactivated CD concentrations of >50 ng ml⁻¹ induced severe impairment of eye formation, gut miscoiling and craniofacial malformations. At concentrations of >75 ng ml⁻¹, microencephaly, muscular kinking and skeletal defects were observed. The term muscular kinking caused by muscular contraction is used to differentiate from that of skeletal kinking in which the spine itself is affected. In tests with the MAS, severe miscoiling of the gut, pericardial edema and mouth malformations were induced at concentrations of >250 ng ml⁻¹. Additional anomalies found at >900 ng ml⁻¹ were skeletal kinking, moderate craniofacial malformations and microencephaly. The effect of metabolic inactivation of CD on embryo growth is presented in Fig. 5. Addition of the MAS increased embryo growth compared to embryos exposed to CD alone. Embryos exposed to CD and the CO-MAS were 87.1% of FETAX solution control growth.

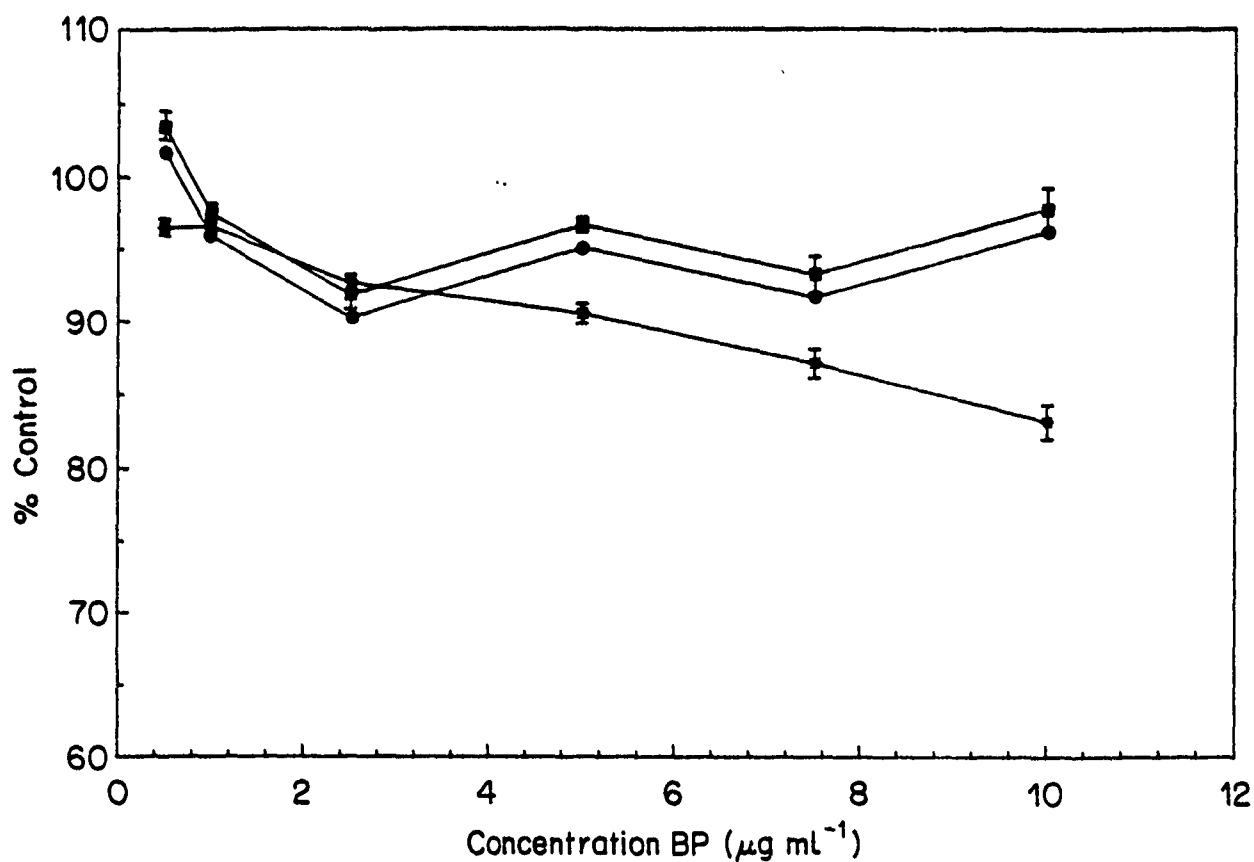


Figure 3. Representative growth curves for final definitive experiments, presented as percent of mean FETAX solution control with SE, for *Xenopus* embryos exposed to unactivated (■) and bioactivated (★) BP for 96 h; (●) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated BP treatments.

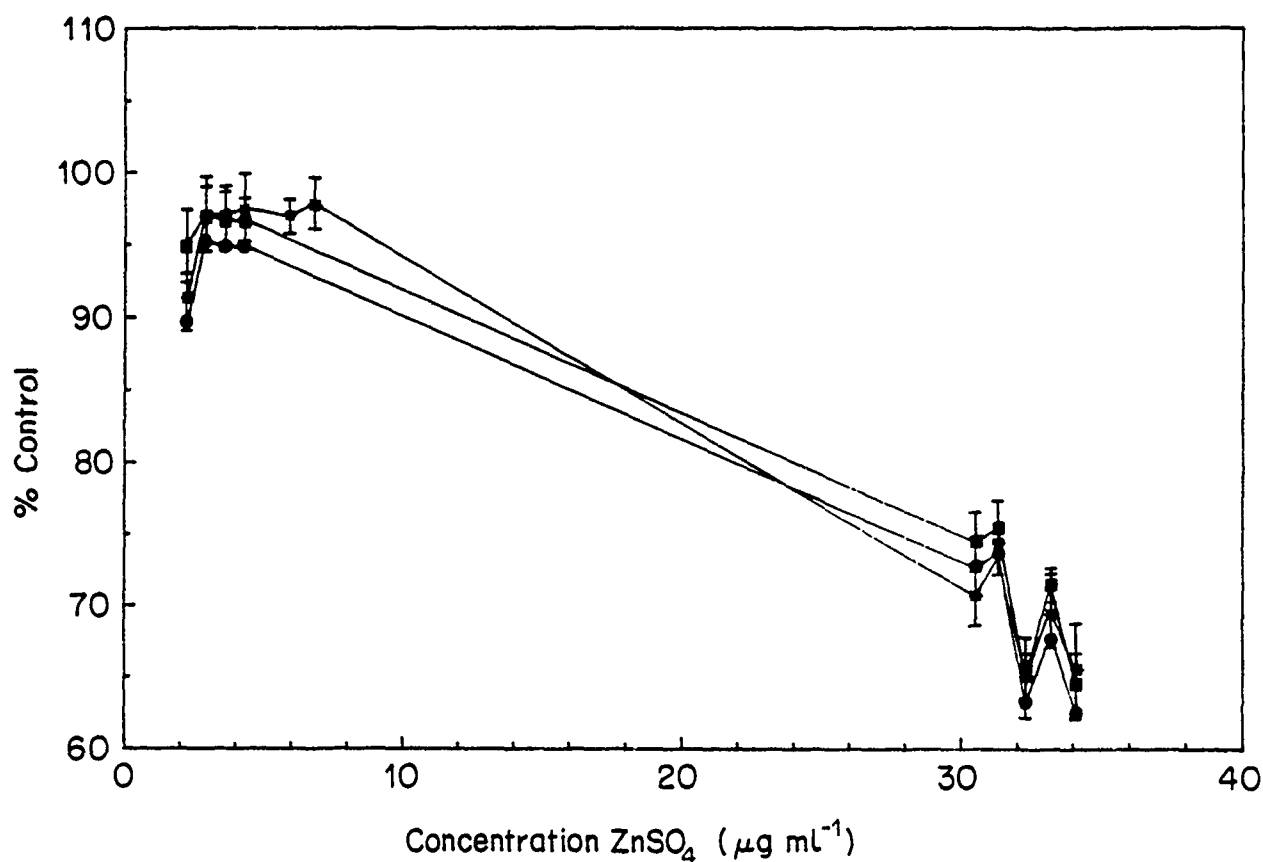


Figure 4. Representative growth curves for final definitive experiments, presented as percent of mean FETAX solution control with SE, for *Xenopus* embryos exposed to unactivated (■) and ZnSO₄ plus the MAS (★) for 96 h; (●) represents the additive growth inhibiting effects of the MAS and unactivated ZnSO₄ treatments.

Table 4. Effect of ZnSO₄ on *Xenopus* embryo development

| Treatment | Unactivated | | Activated | |
|--|-------------|-------------|-------------|-------------|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| FETAX solution control ^a | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 5.0 | 2.5 | 1.3 | 2.5 |
| MAS ^b control ^a | | | | |
| Mortality | - | - | 2.5 | 3.4 |
| Malformation | - | - | 0 | 3.6 |
| 4.0 mg ml ⁻¹ CP ^{a,c} | | | | |
| Mortality | 0 | 0 | 100.0 | 90.0 |
| Malformation | 0 | 0 | - | 100.0 |
| CON-MAS ^d + 85 µg ml ⁻¹ ZnSO ₄ ^{a,f} | | | | |
| Mortality | 0 | 0 | 0 | 0 |
| Malformation | 100.0 | 100.0 | 100.0 | 100.0 |
| LC ₅₀ ^g (µg ml ⁻¹) | 35.0 | 33.8 | 40.0 | 33.4 |
| | (33.9-36.1) | (33.2-33.4) | (39.7-40.3) | (32.7-34.1) |
| LC ₅₀ (µg Zn µg ⁻¹ protein) | - | - | 1.72 | 1.39 |
| | | | (1.72-1.75) | (1.36-1.42) |
| EC ₅₀ ^g (µg ml ⁻¹) | 2.22 | 3.15 | 2.92 | 2.85 |
| | (1.79-2.76) | (2.80-3.54) | (2.55-3.35) | (2.64-3.09) |
| TI ^h | 15.8 | 10.7 | 13.7 | 11.7 |

^a Data presented as percent effect.^b Metabolic activation system.^c Cyclophosphamide.^d Carbon monoxide-inactivated MAS.^e Concentration not normalized to 100 µg ml⁻¹ hardness (as CaCO₃).^f Unactivated column represents data for embryos exposed to 85 µg ml⁻¹ Zn alone.^g Determined by Litchfield-Wilcoxon probit analysis with 95% confidence interval in parentheses. Concentrations normalized to 100 µg ml⁻¹ hardness (as CaCO₃)^{14,15}.^h 96-h LC₅₀/96-h EC₅₀ (malformation).

DISCUSSION

Metabolic activation increased the developmental toxicity of 2-AAF, RA and BP, reduced the effect of CD and had no significant effect on ZnSO₄. Generally, in FETAX, TI values of <1.5 indicate low teratogenic potential whereas higher values signify an increase in the potential hazard.^{4,5,19} However, types and severity of malformations, growth inhibition and results with CO-MAS are also considered. Some compounds with TI values of <1.5 produce severe malformations of major organ systems. These compounds may still pose a hazard for the developing embryo (possibly as an embryo toxin). CO-MAS controls provide the much needed information for assessing the role of P-450-mediated metabolism in teratogenesis.

Even without activation, 2-AAF is a significant teratogenic hazard in FETAX. Several additional factors suggest the importance of biotransformation in 2-AAF teratogenesis. First, activated 2-AAF induced different types of terata with greater severity than unactivated 2-AAF produced. Second, embryos exposed to 2-AAF and the CO-MAS exhibited similar malformation responses, including growth reduction to

embryos exposed to unactivated 2-AAF. Metabolic activation of 2-AAF primarily caused severe malformations of the brain, eye and skeletal system in *Xenopus*. These malformations were not observed in unactivated treatments. Activation also caused a slight decrease in embryo growth and was not as marked as the rates for RA and BP. These anomalies are similar to those found in *in vivo* and *in vitro* mammalian teratogenesis test systems. Izumi²⁰ produced primarily skeletal malformations by administering 2-AAF to mice between the 8th and 15th day of gestation. Faustman-Watts *et al.*²¹ exposed cultured whole-rat embryos to 2-AAF, activated 2-AAF and two metabolites—*N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) and *N*-acetoxy-2-acetylaminofluorene (N-AAF). No malformations and minimal decreases in viability and growth were observed with unactivated 2-AAF at concentrations up to 75 µg ml⁻¹. Concentrations of >60 µg ml⁻¹ bioactivated 2-AAF caused a decrease in embryo viability, incomplete closure of the neural tube and a decrease in embryo growth. In the present study, the concentration of unactivated 2-AAF required to malform all *Xenopus* embryos was 7.5 times greater than activated 2-AAF. Thus, it may be possible that concentrations of >75 µg ml⁻¹ unactivated 2-AAF were required to elicit malformations in cultured

rat embryos. These concentrations were not tested, however. N-OH-AAF and N-AAAF produced ventrolateral protrusion and hypoplasia of the prosencephalon. Although ventrolateral protrusion of the brain was not observed in *Xenopus*, hypoplasia of the rat prosencephalon appears to be similar to microencephaly detected with *Xenopus*.

Bioactivation increased the teratogenic hazard of RA, as indicated by the rise in *TI* and the detrimental effect on embryo growth at concentrations of $>1.0 \text{ mg ml}^{-1}$. Embryos exposed to the CO-MAS and RA developed normally and growth was not inhibited, thus implicating the role of P-450 in teratogenesis. Malformations induced by RA exposure in *Xenopus* are similar to deformities found in mammalian models and possibly humans. Metabolic activation of rifampicin caused severe malformations of the gut, brain, eye and skeletal system at low RA concentrations in *Xenopus*. Oral doses of $>150 \text{ mg kg}^{-1}$ in mice and rats caused spina bifida in both species and cleft palate malformations in the mouse fetus.²² Similar treatment of rabbits had no apparent effect on the developing fetus. Greenaway *et al.*²³ produced open neural tubes

in rats grown *in vitro* and exposed to RA. The response was only observed in the presence of an exogenous microsomal monooxygenase system. Embryo growth was also reduced but was not dependent on the presence of a MAS. In addition, RA has been determined to be more problematic during pregnancy than several other antituberculosis drugs, such as isoniazid and ethambutol.²⁴ Steen and Stainton-Ellis²⁵ reported 9 malformations among 202 exposed newborns (4.5%). The malformations observed were anencephaly (1), hydrocephalus (2), genitourinary anomalies (2), dislocated hip (1) and skeletal reduction deformities (3). However, evaluation of this information is difficult since no information about patient selection was included in the report.²⁶

Exogenous bioactivation increased the potential teratogenic hazard of BP. Activation also significantly decreased *Xenopus* embryo growth at concentrations of $>5.0 \text{ } \mu\text{g ml}^{-1}$. Some of the malformations induced by BP in mammalian test systems are similar to those observed with *Xenopus*. Shum *et al.*²⁷ found that β -naphthoflavone-enhanced BP metabolism in AKR inbred mice injected i.p. with BP between 50 and 300

Table 5. Effect of cytochalasin D on *Xenopus* embryo development

| Treatment | Unactivated | | Activated | |
|---|-------------|-----------|------------|-----------|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| FETAX solution control ^a | | | | |
| Mortality | 3.8 | 2.5 | 1.3 | 1.3 |
| Malformation | 1.3 | 3.8 | 3.8 | 5.1 |
| MAS ^b control ^a | | | | |
| Mortality | - | - | 2.5 | 2.5 |
| Malformation | - | - | 2.6 | 5.2 |
| 1% (v/v) DMSO ^a | | | | |
| Mortality | 2.5 | 6.7 | 0 | 0 |
| Malformation | 5.1 | 3.6 | 5.0 | 5.0 |
| MAS + 1% (v/v) DMSO ^a | | | | |
| Mortality | - | - | 0 | 0 |
| Malformation | - | - | 7.5 | 10.0 |
| 4.0 mg ml ⁻¹ CP ^{a,c} | | | | |
| Mortality | 0 | 3.4 | 100.0 | 100.0 |
| Malformation | 0 | 3.4 | - | - |
| CO-MAS ^d + 1.0 $\mu\text{g ml}^{-1}$ CD ^{a,e} | | | | |
| Mortality | 75.0 | 80.0 | 96.7 | 95.0 |
| Malformation | 100.0 | 100.0 | 100.0 | 100.0 |
| LC ₅₀ ^f (ng ml ⁻¹) | 490 | 433 | 883 | 728 |
| | (376-638) | (313-599) | (765-1020) | (637-831) |
| EC ₅₀ ^f (ng ml ⁻¹) | 121 | 121 | 541 | 551 |
| | (86-170) | (77-199) | (458-638) | (486-625) |
| <i>TI</i> ^g | 4.1 | 3.6 | 1.6 | 1.3 |

^a Data presented as percent effect.

^b Metabolic activation system.

^c Cyclophosphamide.

^d Carbon monoxide-inactivated metabolic activation system.

^e Unactivated column represents data for embryos exposed to 1 $\mu\text{g ml}^{-1}$ CD alone.

^f Determined by Litchfield-Wilcoxon probit analysis with 95% confidence interval in parentheses.

^g 96-h LC₅₀/96-h EC₅₀ (malformation).

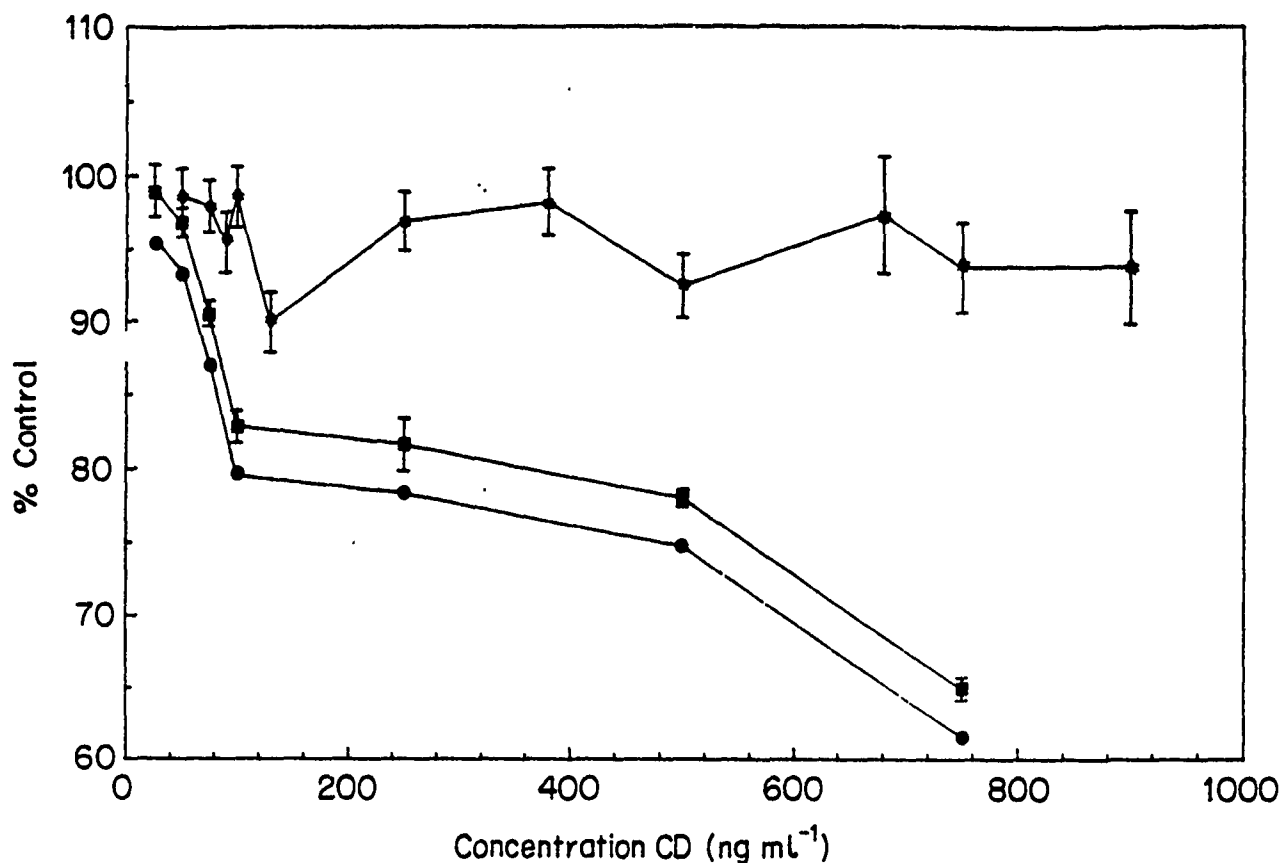


Figure 5. Representative growth curves for final definitive experiments, presented as percent of mean FETAX solution control with SE, for *Xenopus* embryos exposed to unmetabolized (■) and bioinactivated CD (★) for 96 h; (●) represents the additive growth inhibiting effects of the MAS + DMSO and unmetabolized CD treatments.

mg kg⁻¹ was associated with increased *in utero* toxicity and terata (club foot, cleft palate and lip, kinky tail, hemangioma, anophthalmia and scoliosis). Skeletal kinking in *Xenopus* may bear some relationship to skeletal limb defects in mammals.¹³ Similar terata were also observed in B6²⁷ and C57BL/6²⁸ strain mice after i.p. injection, but occurred more frequently than in the AKR strain. Greater incidence of anomalies found in the B6 strain has been attributed to genetic variability in the rate of BP biotransformation [i.e. C57BL/6N and B6 strain mice have a highly inducible P-450 isozyme (AAH) responsible for BP metabolism].

Metabolic activation did not significantly change the developmental toxicity of ZnSO₄, as indicated by the similarity of the *TI* values. Types of malformations induced in activated and inactivated experiments were similar as well. Differences in the *LC*₅₀ values for activated experiments initially suggested that some other factor, such as microsomal metallothioneine, might be responsible for the slight detoxification of ZnSO₄. However, normalization for microsomal protein did not significantly change the variability between the two experiments. If detoxification was the result of metal-protein binding, normalization should correct this variation. Thus, differences were probably due only to genetic variability of the embryos used and minor procedural variations. Growth, which is often the most sensitive endpoint measured by FETAX, was not significantly reduced upon activation. Much emphasis placed on MAS studies concerns the ability to detect only proteratogens. However, a successful MAS must not alter the results of direct-acting terato-

gens. Established direct-acting teratogens, such as ZnSO₄ in *Xenopus*, should be tested in parallel with a metabolic activation system to show that results are not affected. Eventually, all compounds selected for validation of *in vitro* teratogenesis screening systems should be tested with and without the MAS.

Results from experiments performed with the MAS indicate that the developmental toxicity of CD was reduced. Embryos exposed to metabolically inactivated CD developed markedly better, which was reflected in the absence of several different types of terata observed in unactivated CD and in embryo growth. In general, the severity of malformations induced by inactivated CD was significantly less than that caused by unactivated CD. Previous studies with CD in other animal models are similar to results obtained with FETAX. Shepard and Greenaway²⁹ found that C-57 and BALB-C, but not Swiss-Webster, strain mice injected on days 7 through 11 produced exencephaly, hypognathia and skeletal reduction effects. Exencephaly was found to be the predominant malformation in hamsters.³⁰ In the rat, CD was non-teratogenic *in vivo*; however, impaired neural tube closure was observed *in vitro*.³¹ Interestingly, the majority of *Xenopus* embryos that died at concentrations of >2.5 µg ml⁻¹ were arrested during neurulation. The addition of a MAS to the cultured whole-rat embryo system caused significant inhibition of CD teratogenesis.²⁹ Inhibition of teratogenesis was reduced with the addition of CO. In FETAX, CO-MAS restored much of the developmentally toxic effects of CD, including growth inhibition. Based on the present evaluation scheme

using the *TI*, inactivated CD has the potential to be developmentally hazardous. However, differences in the types and severity of malformations and inhibition of growth reduction upon inactivation, as well as reversal of the effects by CO, indicate that CD teratogenesis may be inhibited by the P-450 system. These results suggest the importance of *in vitro* developmental toxicity test systems, including FETAX, in evaluating discrepancies between *in vivo* and *in vitro* models.

Without the ability to metabolize xenobiotics, it is doubtful that *in vitro* test systems will be suitable for screening human developmental toxicants. Results of tests performed with CP,⁴ nicotine,⁵ 2-AAF, RA, BP, Zn and CD all indicate that with an exogenous MAS, FETAX may be able to overcome metabolic incompetence and successfully serve as a screen in developmental toxicity hazard assessment. Such a system should also provide information on the role of MFO metabolism in teratogenesis. FETAX cannot pharmacologically represent the fetal-maternal system of mammals, however it may provide an opportunity to compare the developmental toxicity of parent

compounds and metabolites. By implementing a MAS into the FETAX protocol, we may increase the predictive value by decreasing the number of false-positive and false-negative test results.

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**FURTHER VALIDATION OF FETAX: EVALUATION OF THE
DEVELOPMENTAL TOXICITY OF FIVE KNOWN MAMMALIAN
TERATOGENS AND NON-TERATOGENS**

John A. Bantle¹, Douglas J. Fort, James R. Rayburn,
Donna J. DeYoung and Shirley J. Bush
Department of Zoology, Oklahoma State University,
Stillwater, OK 74078 USA

ABSTRACT

The developmental toxicity of five compounds was evaluated with the Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Late Xenopus laevis blastulae were exposed to 5-azacytidine, methotrexate, pseudoephedrine, aspartame, and amaranth for 96 h. Three separate static-renewal assays were conducted for each compound. Based on Teratogenic Index [LC50/EC50 (malformation)] values, types and severity of induced malformations, and embryo growth, 5-azacytidine and methotrexate tested as having strong teratogenic potential. Pseudoephedrine scored as having moderate teratogenic potential, but amaranth and aspartame had little or no teratogenic potential. Results support the use of FETAX for the screening of developmental toxicants.

INTRODUCTION

Recent attempts to minimize cost and time restraints associated with mammalian teratogenesis assay systems have resulted in the establishment of several alternative test systems utilizing lower life

¹To whom correspondence should be addressed.

forms.¹ One alternative is the Frog Embryo Teratogenesis Assay: Xenopus (FETAX).

FETAX is a 96-h, static-renewal assay capable of screening potential developmental toxicants in a time- and cost-effective manner.² FETAX also has a high degree of developmental relevance to mammals.

Previous studies have demonstrated the utility and versatility of FETAX in the rapid determination of the potential developmental toxicity of pure compounds³⁻⁵ and complex mixtures.⁶⁻⁸ In addition, a successful metabolic activation system (MAS) has been developed⁹⁻¹⁰ evaluated¹¹⁻¹², and applied to the study of toxicological mechanisms of teratogenesis.¹²⁻¹⁴

In this report, we evaluate the developmental toxicity of five compounds using FETAX.

MATERIALS AND METHODS

Adult animal care, breeding and embryo collection were performed according to the method of Dawson and Bantle.⁴

Sets of 25 embryos were placed in 60-mm covered glass Petri dishes with varying concentrations of the appropriate test compound (Sigma Chemical Co., St. Louis, MO) dissolved in FETAX solution.⁴ For each compound 8 to 16 concentrations were tested in duplicate. Four separate control dishes of 25 embryos

each were exposed to FETAX solution alone. Treatment and control dishes contained a total of 8 ml of solution. Antibiotics (100 U/ml penicillin G-0.1 mg/ml streptomycin sulfate)¹⁴ were used in the final test with aspartame to control bacterial growth.

For each compound at least one range and three definitive dose-response tests were conducted. All compounds except for amaranth were subjected to blind testing. In this testing format, the samples were coded and given to the technician who had no knowledge of what they contained. The technician collected and analyzed all data. Since amaranth in solution was clearly red, it was not included in the blind battery of compounds to minimize potential bias. The pH of all test compounds tested was 7.0 to 7.5. Embryos were cultured at $24 \pm 1^{\circ}\text{C}$ throughout the test.

Solutions were renewed every 24 h of the four d tests and any dead animals removed. At 96 h, surviving embryos were fixed in 3.0% (w/v) formalin. The number of dead, and malformed survivors, as well as developmental stage¹⁵ were determined using a dissecting microscope.

For each test, probit analysis¹⁶ was used to determine the 96-h LC50 (median lethal concentration), 96-h EC50 (concentration inducing gross terata in 50%

of the surviving embryos), and 95% percent confidence intervals. A Teratogenic Index [TI=LC50/EC50 (malformation)], useful in assessing levels of teratogenic potential^{3-5,9-13}, was also determined.

Head-tail length (growth) was measured using an IBM-compatible computer equipped with digitizing software (Jandel Scientific, Corte Madera, CA). For each test, the minimum concentration to inhibit growth (MCIG) was calculated using the t-Test for grouped observations ($p < 0.05$).

RESULTS

Results from the FETAX tests are presented in Table 1. It was not possible to determine and EC50 for the second pseudoephedrine test although the value was between 200 and 250 mg/l. This would yield a TI of approximately 1.9 for this test. Representative concentration-response and growth-inhibition curves for the five compounds are illustrated in Figures 1 and 2, respectively. In this investigation, the FETAX solution control mortality and malformation rates were 111 of 1500 (7.4%) and 116 of the 1389 survivors (8.4%), respectively. Acceptable rates of control mortality and malformation are generally $\leq 10\%$.

Malformations induced by amaranth were limited to miscoiling of the gut and pericardial edema at

TABLE 1.
Developmental Toxicity of Five Compounds Tested with FETAX.

| Compound | Test # | LC50 ^a | EC50 ^a | TI | MCIG ^b | MCIG ^c |
|-----------------|----------------|------------------------|------------------------|-------|-------------------|-------------------|
| Aspartame | 1 | >10000 ^e | >10000 ^e | NA | 3000 | NA |
| Aspartame | 2 | >10000 ^e | >10000 ^e | NA | 7000 | NA |
| Aspartame | 3 ^d | 13920 (11928-16234) | 13140 (10951-15788) | 1.1 | 7000 | 50 |
| Amaranth | 1 | 2670 (2130-3250) | 3530 (3030-5170) | 0.8 | 4000 | 150 |
| Amaranth | 2 | 3680 (3470-3910) | 3060 (2920-3210) | 1.2 | 1000 | 82 |
| Amaranth | 3 | 3810 (3608-4020) | 3910 (3824-3999) | 1.0 | 3750 | 98 |
| Pseudoephedrine | 1 | 440 (394-480) | 260 (214-326) | 1.7 | 200 | 45 |
| Pseudoephedrine | 2 | 420 (407-428) | 200<EC50<250 | -1.9 | 200 | 48 |
| Pseudoephedrine | 3 | 390 (387-401) | 210 (205-216) | 1.9 | 150 | 38 |
| Methotrexate | 1 | 508 (475-543) | 22 (21-24) | 23.1 | 20 | 4 |
| Methotrexate | 2 | 500 (485-511) | 30 (29-33) | 16.7 | 10 | 2 |
| Methotrexate | 3 | 230 (1-324) | 20 (9-34) | 11.5 | 30 | 13 |
| 5-Azacytidine | 1 | 620 (587-658) | >50 | <12.4 | 400 | 65 |
| 5-Azacytidine | 2 | 600 (563-643) | 70 (62-72) | 8.6 | 100 | 16 |
| 5-Azacytidine | 3 | 430 (382-469) | 20 (19-24) | 21.5 | 70 | 16 |

^a mg/l with (95% confidence interval).

^b Minimum Concentration to Inhibit Growth as mg/l.

^c Minimum Concentration to Inhibit Growth as a Percent of LC50.

^d With 100 U/ml Penicillin and 100 ug/ml Streptomycin.

^e Maximum soluble concentration in FETAX solution.

NA Not available.

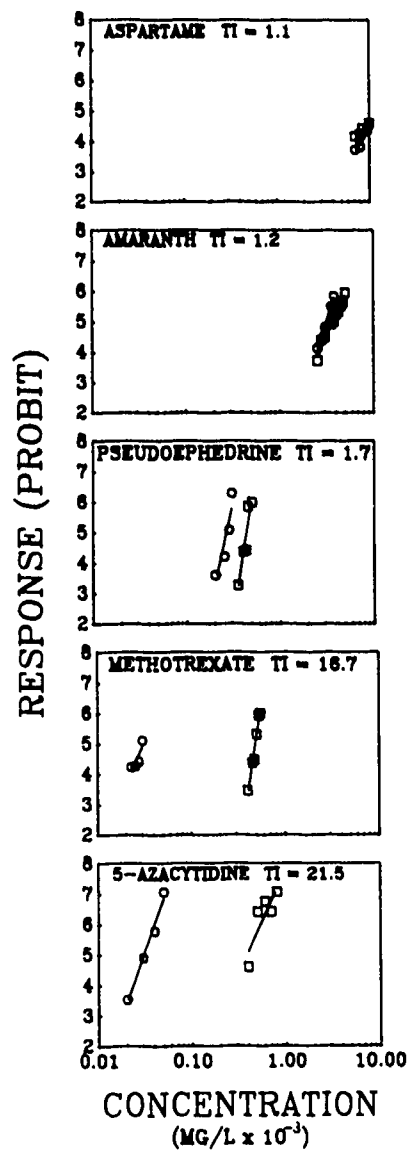


FIGURE 1. Representative concentration-response curves and respective Teratogenic Index values for the five compounds tested with FETAX.

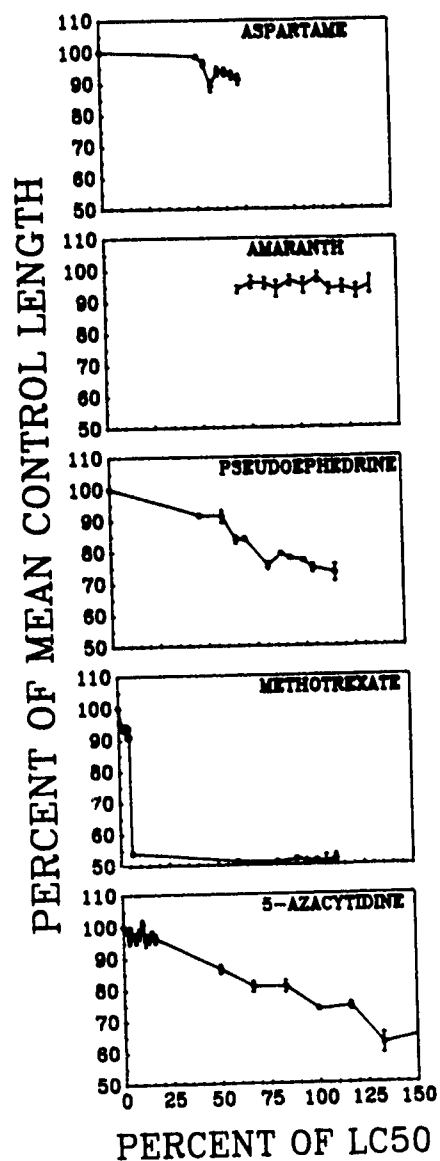


FIGURE 2. Representative embryo growth curves for the five compounds. Concentrations are expressed as percent of the respective compound LC50. Growth is expressed as percent of mean FETAX solution control length \pm SEM.

concentrations >2500 mg/l and 4500 mg/l, respectively. Concentrations >6000 mg/l caused miscoiling of the gut, craniofacial defects and mouth deformities. Muscular kinking was observed at concentrations exceeding 9000 mg/l. Test #1 and #2 were conducted without antibiotics. Addition of the penicillin-streptomycin mixture did not affect the types or the severity of the induced malformations.

At concentrations >200 mg/l, pseudoephedrine produced miscoiling of the gut and skeletal kinking. Exposure to concentrations >250 mg/ml induced pericardial and craniofacial edema, as well as microphthalmia. Abnormal development of the mouth and blistering of the dorsal fin were noted at concentrations exceeding 280 mg/l.

Methotrexate produced miscoiling of the gut and microphthalmia above 20 mg/l. Exposure to concentrations >30 mg/l induced microencephaly and hydroencephaly, as well.

Primary malformations associated with exposure to >30 mg/l 5-azacytidine were miscoiling of the gut and incomplete development of the mouth. Concentrations >70 mg/l produced muscular kinking, visceral edema, and microencephaly. Microphthalmia and craniofacial defects were also observed above 100 mg/l.

DISCUSSION

In FETAX, teratogenic potential is determined by the TI values, embryo growth, and the type and severity of induced malformations. Generally, TI values <1.5 indicate low teratogenic potential and higher values signify an increase in the potential hazard.^{3-5,9-11} Higher TIs indicate a larger separation of the mortality and malformation dose-response curves, and therefore, a greater potential for the exposed embryos to be deformed in the absence of lethality.⁴ Amaranth and aspartame possess low TI values, thus showing little separation in mortality and malformation curves presented as Figure 1. Pseudoephedrine, methotrexate, and 5-azacytidine represent compounds with increasing separation in the curves, and thus increasing TI values. However, it is also necessary to consider the types and severity of terata, since all chemicals are potential teratogens if administered in appropriate doses at sensitive stages of development.¹⁷ Compounds with TI values <1.5 may still pose a hazard to developing organisms, possibly as embryotoxins.

The MCIG (expressed as % compound LC50) has also proven useful in assessing developmental toxicity.^{3-5,11} Dawson et al.⁵ suggest that compounds possessing

significant teratogenic potential generally inhibit growth at concentrations <30% of the respective LC50 values. Both 5-azacytidine and methotrexate inhibited growth at <30% of the LC50, whereas pseudoephedrine, aspartame, and amaranth caused inhibition between 38% and 150% of each particular LC50. In addition, rates of growth inhibition (i.e. slope) and the overall reduction in embryo growth increase with the TI value. Growth-inhibition curves presented as Figure 2 reflect this phenomenon.

Evaluation based on this method suggest that methotrexate, 5-azacytidine, and, possibly, pseudoephedrine have the potential to be teratogenic. Amaranth and aspartame produced low TI values, had no profound effect on embryo growth, and induced relatively few anomalies only at high concentrations. Thus, amaranth or aspartame do not pose a teratogenic hazard.

Results from this study are in agreement with current mammalian literature, with the possible exception of pseudoephedrine. Although surveys¹⁸ have reported cases of malformed human infants born to mothers using decongestants containing pseudoephedrine, results are clouded by the simultaneous administration of multiple other drugs,

including foam contraceptives. Thus, pseudoephedrine has not been conclusively shown to be a human teratogen.^{19,20} We are currently testing pseudoephedrine with metabolic activation system⁹⁻¹¹ to determine if the positive results of the in vitro assay may be attributed to the lack of maternal metabolism.

Neither amaranth²¹⁻²³ nor aspartame²⁴⁻²⁶ has been shown to be teratogenic in mammals or humans. Malformations observed in mammalian systems with 5-azacytidine and methotrexate are similar to those observed in Xenopus in this study. 5-azacytidine has been shown to induce exencephaly, anencephaly, and eye defects in both rat²⁷ and mouse²⁸ fetuses. Brain malformations have also been observed in rats subjected to azaguanine.²⁹ The concentration-response data for 5-azacytidine proved to be somewhat variable and it was not possible to generate an EC50(malformation). However, all tests performed indicated that 5-azacytidine was a strong teratogen. Methotrexate exposure caused skeletal, palate, and brain anomalies in mammals.³⁰⁻³¹ Defects observed in humans include absence of frontal bones and digits, as well as, premature cranio-synostosis.³²

Similarities in the results of aspartame tests conducted with and without penicillin-streptomycin suggested that no potentiation or antagonism of developmental toxicity was caused by antibiotic supplementation.

Results of FETAX tests with these five compounds indicated 5-azacytidine and methotrexate have strong teratogenic potential. Pseudoephedrine has the potential to be teratogenic, but results were not as convincing as those for 5-azacytidine and methotrexate. Amaranth and aspartame displayed little or no teratogenic potential. Including this study, FETAX tests in this laboratory on 28 compounds have produced six false results (79%).^{3-5,33} With the exogenous metabolic activation system, FETAX has correctly predicted 12 of 13 compounds tested (92%)⁹⁻¹³, for a combined predictive accuracy of 89%. Results obtained warrant further evaluation of FETAX for the rapid screening of developmental toxicants.

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The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985).

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**Synergism and Antagonism Induced by Three Carrier
Solvents with t-Retinoic Acid and 6-Aminonicotinamide using
FETAX**

J.R. Rayburn¹, D.J. Fort¹, R. McNew², and J.A. Bantle¹

¹Department of Zoology, Oklahoma State University,
Stillwater, Oklahoma 74078, USA. ²Agricultural
Statistics Laboratory, University of Arkansas,
Fayetteville, Arkansas 72701, USA.

The large number of water-insoluble chemicals requiring toxicity testing necessitates the development, validation and use of chemical cosolvents. Carrier solvents (cosolvents), such as dimethylsulfoxide (DMSO), acetone, and triethylene glycol (TG), are commonly used to solubilize hydrophobic compounds (Yalkowsky 1981). However, the use of solvents with in vitro bioassays may alter the developmental toxicity of test materials. Solvents interact with other compounds to change rates of reactions, membrane potentials, mutagenic activity, and many other cell processes (Coetzee and Ritchie 1969; Nemethy 1986; Demey et al. 1983; Gichner and Veleminsky 1987). For this reason solvent-compound interaction studies were performed to determine if the developmental toxicity of test materials was altered.

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), formally described by Dumont et al. (1983), is a 96-hr bioassay which determines relative teratogenic hazard. Several labs have evaluated compounds as well as environmental mixtures with FETAX (Courchesne and Bantle 1985; Dawson and Bantle 1987a; Dumont et al. 1983; Sabourin and Faulk 1987; Dawson et al. 1985). Fort et al. (1989) have also developed and evaluated an exogenous metabolic activation system for FETAX. The purpose of these experiments was to determine whether carrier solvents interacted with the teratogens t-retinoic acid and 6-aminonicotinamide to affect survival, development and growth of *Xenopus laevis* embryos.

MATERIALS AND METHODS

Animal care and breeding were performed according to Bantle et al. (1989). FETAX solution (Dawson and Bantle 1987b) which is composed of 625 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg CaSO₄ · 2H₂O, and 75 mg MgSO₄ per L was used as the diluent for all experiments. For each concentration-response test, two groups of 25 embryos each were placed in 60 X 15 mm glass Petri dishes containing a total of 10 mL of solution. Four groups of 25 embryos each were used as controls for each test. Each experiment followed standard methods of test operation and embryo evaluation according to Bantle et al. (1989).

One range and three definitive experiments determined the 96-hr LC50, 96-hr EC50 (malformation), Teratogenic Index (TI) (96-hr LC50/ 96-hr EC50) and Minimum Concentration to Inhibit Growth (MCIG) for three solvents and two teratogens. The solvents were dimethylsulfoxide (DMSO) (CAS# 67-68-5; Sigma Chemical Co., St. Louis, Missouri)

Send correspondence to Dr. J.A. Bantle at address above.

acetone (CAS# 67-64-1; Fisher Scientific, Houston, Texas), and triethylene glycol (TG) (CAS# 112-27-6; Aldrich Chemical Co., Milwaukee, Wisconsin). The teratogens obtained from Sigma were trans-retinoic acid (RA) (CAS# 302-79-4) and 6-aminonicotinamide (6-AN) (CAS# 329-89-5). The 96-hr LC25 and LC50, and 96-hr EC25 and EC50 (malformation) were determined using the Litchfield-Wilcoxon probit analysis computer program part of the the Manual of Pharmacologic Calculations (Tallarida and Murray 1980). Dunnett's test was used to determine the MCIG and No Observable Effect Concentrations (NOEC). This data was used in determining test concentrations for the solvent-compound interaction study.

Two concentrations for each solvent and test material were selected. The two solvent concentrations were the NOEC and 96-hr EC25. The NOEC is the highest cosolvent concentration used in FETAX. The 96-hr EC25 was chosen in order to positively identify any interactions that might be taking place that were not observable at lower concentrations. The 6-AN and RA concentrations were the 96-hr EC25 and 96-hr LC25. These concentrations allowed for examination of the effects on both malformation and mortality. Both 6-AN and RA were soluble at these concentrations without the solvents.

The interaction experiments contained individual treatments for all concentrations and the interaction treatments for one solvent and one teratogen per experiment. The nine treatments were: 1) FETAX solution controls, 2) EC25 of teratogen, 3) LC25 of teratogen, 4) NOEC level of solvent, 5) EC25 of solvent, 6) EC25 of teratogen & NOEC of solvent, 7) EC25 of teratogen & EC25 of solvent, 8) LC25 of teratogen & NOEC of solvent, and 9) LC25 of teratogen & EC25 of solvent. All experiments contained four replicates of 25 embryos each per treatment. The analysis of solvent-teratogen interactions represented three pooled experiments using three different breeding pairs. Every 24 hrs dead embryos were removed and solutions changed. After 96 hrs of exposure, embryos were anesthetized with 3-Aminobenzoic Acid Ethyl Ester (MS-222) (CAS# E1-052-1; Sigma) and the number malformed recorded. The larvae were then killed with 0.7% (w/v) formalin so that head-tail length (growth) could be measured.

ANOVA was used to determine differences from theoretical additive values for mortality, malformation and growth according to Steel and Torrie (1980). Values were determined by partitioning the sum of squares for the interactions on mortality and malformation using the statistical software systat (Wilkinson 1989). Growth interactions were determined in the same manner with the Statistical Analysis System (SAS). Effects on the TI were inferred from the shifts of the mortality and malformation curves.

RESULTS AND DISCUSSION

Compounds were considered to pose teratogenic hazard when the TI > 1.5 (Bantle et al. 1989; Dawson et al. 1989). The TI represents the separation between the mortality and malformation curves. If a synergistic or antagonistic response altered a TI, false conclusions regarding teratogenic hazard could result.

Preliminary work revealed that all solvents caused effects at 2.0% (v/v) concentrations. Acetone had the highest TI of the solvents followed by DMSO and then TG (Table 1). NOEC levels for DMSO, Acetone and TG were 1% (v/v) for malformation, 0.9% (v/v) for malformation, and 1.7% (v/v) for growth, respectively. The 96-hr EC25 (malformation) for DMSO, Acetone and TG were 1.2% (v/v), 1.0% (v/v), and 2.0% (v/v).

Both 6-AN and RA caused teratogenic effects in FETAX and 6-AN had a much higher TI (Table 1). Mammalian literature supports the classification of RA and 6-AN as teratogens (Schardein et al. 1967; Chamberlain 1966; Kochhar 1975; Lammer et al. 1985). The 96-hr

EC25 (malformation), and 96-hr LC25 for each teratogen were as follows: Retinoic acid was 0.02 mg/L and 0.25 mg/L; 6-aminonicotinamide was 2 mg/L and 2500 mg/L.

Table 1. 96-hr LC50, 96-hr EC50(malformation), Teratogenic Index (TI)^a, and Minimum Concentration to Inhibit Growth (MCIG) for Dimethylsulfoxide (DMSO), Acetone, Triethylene glycol (TG), Retinoic Acid (RA) and 6-aminonicotinamide (6-AN).

| Compound | Trial | 96-hr LC50 | 96hr EC50 | TI | MCIG |
|----------------------|-------|-------------------------------|--------------------|------|------|
| DMSO ^b | 1 | 1.81(1.75-1.87 ^c) | 1.4 (1.32-1.48) | 1.3 | 1.3 |
| | 2 | 1.77(1.61-1.95) | 1.29(1.25-1.33) | 1.4 | 1.7 |
| | 3 | 1.86(1.4-2.3) | 1.24(0.83-1.8) | 1.5 | 1.5 |
| Acetone ^b | 1 | 2.16(2.07-2.25) | 1.4 (1.29-1.43) | 1.6 | 1.8 |
| | 2 | 2.49(2.10-2.95) | 1.4 (1.04-1.36) | 1.8 | 1.5 |
| | 3 | 1.92(1.90-2.14) | 1.06(0.91-1.17) | 1.83 | 1.0 |
| TG ^b | 1 | 2.4 (2.02-2.85) | 2.0 (2.01-2.13) | 1.2 | 1.8 |
| | 2 | 2.75(2.70-2.82) | 2.4 (2.37-2.45) | 1.1 | 1.8 |
| | 3 | 2.19(2.19-2.32) | 2.05(1.99-2.11) | 1.07 | 1.7 |
| RA ^d | 1 | 0.25(0.22-0.28) | 0.024(0.018-0.031) | 10.4 | 0.06 |
| | 2 | 0.50(0.46-0.61) | 0.044(0.032-0.060) | 11.4 | 0.08 |
| 6-AN ^{d e} | 1 | 3190(3000-3400) | 5.3(2.5-7.5) | 602 | 100 |
| | 2 | 2950(2800-3100) | 5.7(5.3-6.2) | 518 | NA |

^a TI = 96-hr LC50/96-hr EC50 (malformation).

^b Concentrations expressed as % (v/v).

^c 95% confidence limits.

^d Concentrations expressed as mg/L.

^e Data from Dawson et al. (1989).

Tables 2 and 3 show the treatment effects for each of the six solvent interaction experiments. Control malformation and mortality generally were less than 8% except for two experiments (Tables 2 and 3). Table 3 revealed that 2500 mg/L 6-AN was not the LC25 but was actually closer to the LC50. The reason for these discrepancies may be attributed to an antibiotic used by Dawson et al. (1989) to control bacterial growth and in the present study no antibiotic treatments were used. Because the values for solvent and compounds often varied due to different breeding pairs, the values were kept discrete for each experiment. For example, DMSO at 1.2% (v/v) should have caused 25 percent malformation. The retinoic acid experiment (Table 2) showed 20.9% malformation for 1.2% (v/v) DMSO, and the 6-aminonicotinamide (Table 3) showed 28.3% malformation for 1.2% (v/v) DMSO.

Effects on length were not different from additive values at $p = 0.05$. Therefore, none of the cosolvents affected embryo growth in a synergistic or antagonistic manner.

The interaction results for RA combined with DMSO (Figure 1) showed that mortality increased significantly at $p = 0.001$. DMSO at 1% (v/v) and 1.2% (v/v) concentrations with 0.25 mg/L RA increased mortality by 34.3% and 47.3%, respectively. Because

malformation of DMSO with RA was not significantly different at either concentration, the synergistic effect on mortality should cause the mortality curve to shift to the left (reducing the 96-hr LC50). DMSO addition would reduce the TI for RA.

Table 2. Interactive effects on mortality, malformation, and growth inhibition caused by the solvents and retinoic acid (RA).

| Treatment ^a | Mortality No. (%) | Malformation No. (%) | Mean Length (mm) |
|--------------------------------------|-------------------------------|-------------------------|---------------------|
| FETAX soln. | | | |
| Control | 11 (3.7 ± 1.43 ^b) | 12 (4.2 ± 0.77) | 9.61 ± 0.064 |
| DMSO ^c RA ^d | | | |
| 1.0% | 11 (3.7 ± 0.77) | 29 (10.1 ± 1.53) | 9.44 ± 0.092 |
| 1.2% | 22 (7.3 ± 1.76) | 58 (20.9 ± 0.94) | 9.17 ± 0.064 |
| 0.02 | 18 (6.0 ± 1.15) | 65 (23.0 ± 3.58) | 9.58 ± 0.081 |
| 0.25 | 64 (21.3 ± 6.86) | 236 (100.0) | 7.06 ± 0.381 |
| 1.0% 0.02 | 13 (4.3 ± 1.25) | 104 (36.3 ± 6.06) | 9.15 ± 0.098 |
| 1.2% 0.02 | 15 (5.0 ± 1.31) | 111 (38.9 ± 3.61) | 9.10 ± 0.069 |
| 1.0% 0.25 | 167 (55.7 ± 7.95) | 133 (100.0) | 6.55 ± 0.305 |
| 1.2% 0.25 | 217 (72.3 ± 9.84) | 83 (100.0) | 6.25 ± 0.460 |
| FETAX soln. | | | |
| Control | 6 (2.0 ± 0.78) | 14 (4.7 ± 1.54) | 9.54 ± 0.046 |
| Acetone ^c RA ^d | | | |
| 0.9% | 9 (3.0 ± 1.22) | 31 (10.6 ± 1.62) | 9.04 ± 0.061 |
| 1.0% | 13 (4.3 ± 1.25) | 65 (22.7 ± 1.12) | 8.94 ± 0.087 |
| 0.02 | 12 (4.0 ± 1.21) | 93 (32.2 ± 5.49) | 9.17 ± 0.087 |
| 0.25 | 87 (29.0 ± 7.45) | 213 (100.0) | 6.33 ± 0.352 |
| 0.9% 0.02 | 17 (5.7 ± 1.04) | 145 (51.1 ± 5.18) | 8.89 ± 0.066 |
| 1.0% 0.02 | 18 (6.0 ± 2.25) | 251 (89.2 ± 4.94) | 8.61 ± 0.098 |
| 0.9% 0.25 | 132 (44.0 ± 9.86) | 168 (100.0) | 6.20 ± 0.211 |
| 1.0% 0.25 | 120 (40.0 ± 11.79) | 180 (100.0) | 6.11 ± 0.140 |
| FETAX soln. | | | |
| Control | 23 (7.7 ± 2.9) | 22 (7.9 ± 0.67) | 9.36 ± 0.061 |
| TG ^c RA ^d | | | |
| 1.7% | 35 (11.7 ± 2.53) | 58 (22.1 ± 2.68) | 8.28 ± 0.144 |
| 2.0% | 27 (9.0 ± 2.04) | 94 (34.5 ± 2.76) | 8.31 ± 0.234 |
| 0.02 | 20 (6.7 ± 2.22) | 68 (24.5 ± 1.62) | 9.17 ± 0.061 |
| 0.25 | 54 (18.0 ± 4.89) | 246 (100.0) | 7.32 ± 0.237 |
| 1.7% 0.02 | 26 (8.7 ± 2.78) | 100 (36.4 ± 3.72) | 8.43 ± 0.199 |
| 2.0% 0.02 | 25 (8.3 ± 2.78) | 131 (47.7 ± 4.76) | 8.32 ± 0.210 |
| 1.7% 0.25 | 115 (38.3 ± 9.99) | 185 (100.0) | 6.20 ± 0.390 |
| 2.0% 0.25 | 139 (46.3 ± 11.11) | 161 (100.0) | 6.48 ± 0.498 |

^a N for all treatments equaled 300 embryos, 12 dishes of 25 embryos each.

^b Standard Error

^c Concentrations are v/v%.

^d Concentrations are mg/L

TG = Triethylene Glycol

DMSO = Dimethylsulfoxide

RA in the presence of acetone (Figure 1) significantly increased malformation by 38.5%

with 1% (v/v) acetone and 0.02 mg/L RA while not changing the mortality at 1% acetone and 0.25 mg/L RA. The synergistic shift of the malformation concentration-response curve to the left (reducing the 96-hr EC50) would increase the TI with acetone at the 1% (v/v) level. There were no significant effects at the 0.9% (v/v) level (NOEC) of acetone.

Table 3. Interactive effects on mortality, malformation and growth inhibition caused by the solvents and 6-aminonicotinamide (6-AN).

| Treatment ^a | Mortality No. (%) | Malformation No. (%) | Mean Length (mm) |
|--|-------------------------------|-------------------------|---------------------|
| FETAX soln. | | | |
| Control | 12 (4.0 ± 1.39 ^b) | 26 (8.9 ± 2.10) | 9.51 ± 0.115 |
| DMSO ^c 6-AN ^d | | | |
| 1.0% | 14 (4.7 ± 1.69) | 43 (15.0 ± 1.70) | 9.55 ± 0.124 |
| 1.2% | 25 (8.3 ± 2.97) | 78 (28.3 ± 3.11) | 9.60 ± 0.144 |
| 2.0 | 12 (4.0 ± 1.30) | 71 (24.8 ± 1.80) | 9.77 ± 0.064 |
| 2500 | 150 (50.0 ± 5.34) | 150 (100.0) | 6.70 ± 0.063 |
| 1.0% 2.0 | 18 (6.0 ± 1.94) | 94 (33.4 ± 4.41) | 9.71 ± 0.167 |
| 1.2% 2.0 | 19 (6.3 ± 2.43) | 132 (46.9 ± 6.84) | 9.56 ± 0.124 |
| 1.0% 2500 | 300(100.0) | | |
| 1.2% 2500 | 300(100.0) | | |
| FETAX soln. | | | |
| Control | 7 (2.3 ± 1.25) | 15 (5.1 ± 0.74) | 9.72 ± 0.084 |
| Acetone ^c 6-AN ^d | | | |
| 0.9% | 11 (3.7 ± 1.04) | 43 (15.0 ± 1.26) | 9.43 ± 0.072 |
| 1.0% | 20 (6.7 ± 2.16) | 74 (26.6 ± 1.61) | 9.17 ± 0.061 |
| 2.0 | 10 (3.3 ± 1.46) | 70 (24.2 ± 1.23) | 9.76 ± 0.075 |
| 2500 | 167 (55.7 ± 8.76) | 133 (100.0) | 6.06 ± 0.182 |
| 0.9% 2.0 | 25 (8.3 ± 2.89) | 75 (27.2 ± 1.06) | 9.29 ± 0.098 |
| 1.0% 2500 | 11 (3.7 ± 1.04) | 135 (46.8 ± 2.87) | 9.10 ± 0.058 |
| 0.9% 2.0 | 288 (96.0 ± 1.21) | 12 (100.0) | 6.08 ± 0.084 |
| 1.0% 2500 | 294 (98.0 ± 1.15) | 6 (100.0) | 5.84 ± 0.055 |
| FETAX soln. | | | |
| Control | 29 (9.7 ± 2.97) | 17 (6.5 ± 1.45) | 9.44 ± 0.136 |
| TG ^c 6-AN ^d | | | |
| 1.7% | 52 (17.3 ± 6.35) | 40 (16.0 ± 2.42) | 9.00 ± 0.156 |
| 2.0% | 57 (19.0 ± 6.44) | 113 (46.6 ± 4.11) | 8.60 ± 0.202 |
| 2.0 | 26 (8.7 ± 3.18) | 57 (20.9 ± 1.36) | 9.50 ± 0.181 |
| 2500 | 209 (69.7 ± 9.98) | 91 (100.0) | 6.69 ± 0.147 |
| 1.7% 2.0 | 64 (21.3 ± 6.27) | 168 (71.4 ± 7.47) | 8.60 ± 0.240 |
| 2.0% 2.0 | 33 (11.0 ± 5.10) | 218 (81.6 ± 4.14) | 8.40 ± 0.240 |
| 1.7% 2500 | 299 (99.7 ± 0.33) | 1 (100.0) | 7.74 |
| 2.0% 2500 | 300(100.0) | | |

^a N for all treatments equaled 300 embryos, 12 dishes of 25 embryos per dish.

^b Standard Error

^c Concentrations expressed as % (v/v).

^d Concentrations expressed as mg/L.

TG = Triethylene Glycol

DMSO = Dimethylsulfoxide

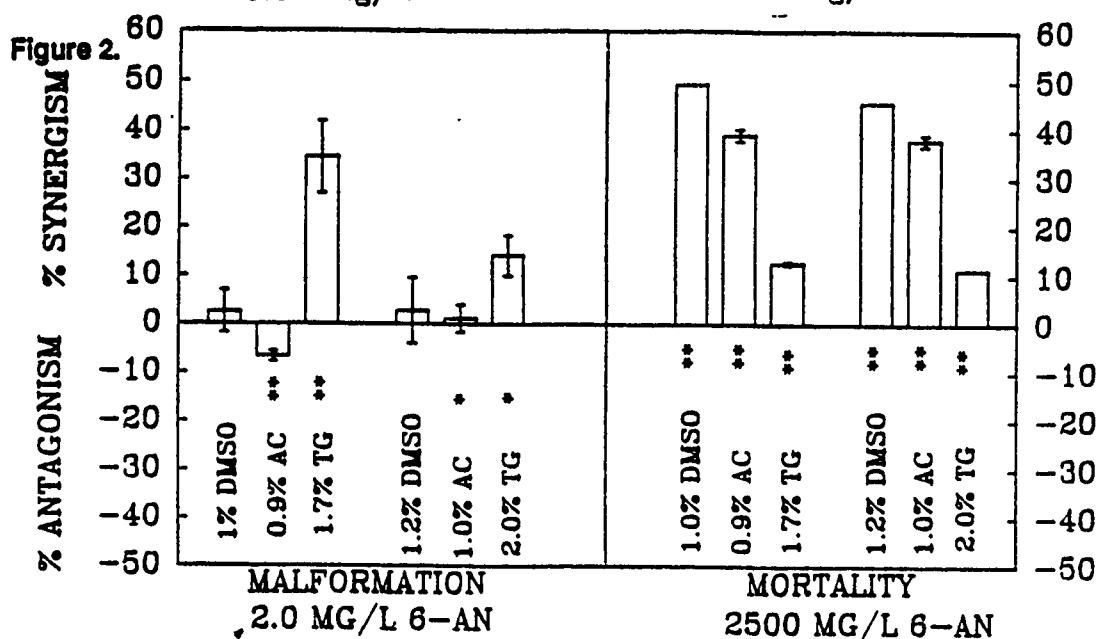
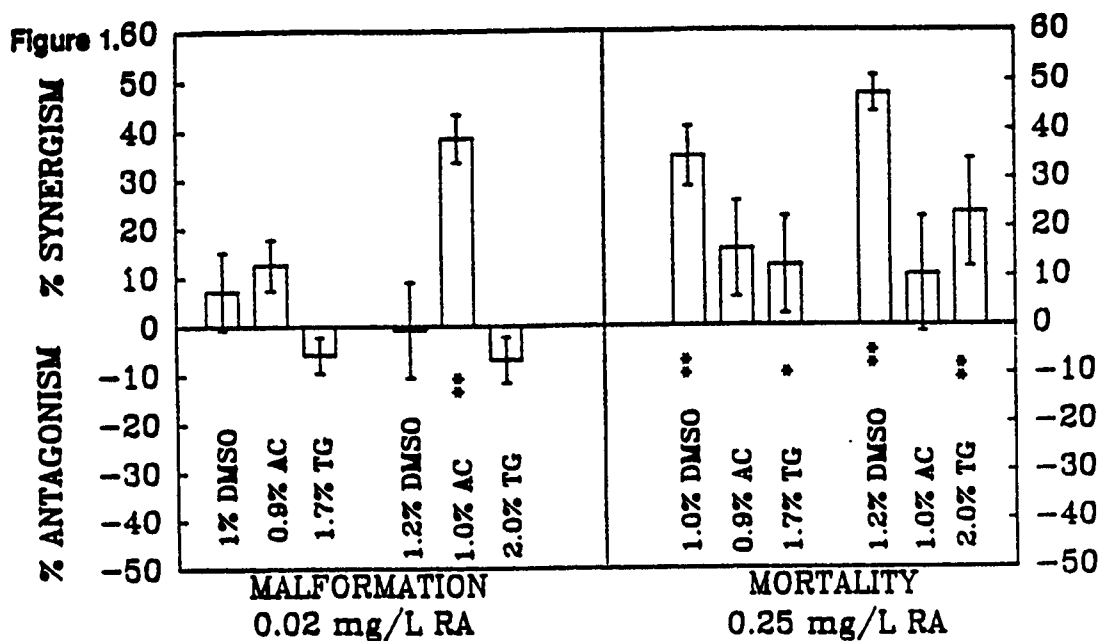


Figure 1. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for retinoic acid (RA).

Figure 2. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for 6-aminonicotinamide (6-AN).

Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at the $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments and is why two close values are significantly different.

TG with RA had effects on both malformation and mortality (Figure 1). Malformation with 2.0% (v/v) TG and 0.02 mg/L RA reduced the additive value by 7% at $p = 0.059$. Mortality rates of 1.7% (v/v) TG and 2.0% (v/v) TG with 0.25 mg/L RA increased the additive values

by 12.3% and 23% respectively. The antagonistic shift of the malformation curve to the right (increasing the 96-hr EC50) and the synergistic shift of the mortality curve to the left (decreasing 96-hr LC50) would produce a reduced TI.

DMSO combined with 6-AN synergistically increased mortality rates at both levels of the solvent at $p = 0.001$ (Figure 2). The increase in mortality for 1% (v/v) DMSO and 1.2% (v/v) with 2500 mg/L 6-AN was 49.3% and 45.7%, respectively. The mortality curve would shift to the left, decreasing the 96-hr LC50 and reducing the TI.

Acetone tested with 6-AN had significant effects at $p \leq 0.05$ for both malformation and mortality (Figure 2). The malformation decreased with 0.9% (v/v) acetone and 2 mg/L 6-AN by 6.8%. However, the 1.0% (v/v) acetone with 2 mg/L 6-AN increased malformation by only 1.2%. The mortality caused by acetone with 6-AN increased for 0.9% (v/v) acetone and 1% (v/v) acetone with 2500 mg/L 6-AN by 39% and 38% respectively. Because the synergistic increase in mortality (reducing the 96-hr LC50) outweighed the antagonistic effects on malformation, the TI should be reduced.

TG in the presence of 6-AN significantly increased both malformation and mortality at $p \leq 0.05$ (Figure 2). TG at 1.7% (v/v) and 2% (v/v) combined with 2 mg/L 6-AN increased malformation by 34.5% and 14.2% respectively. Mortality increased for 1.7% (v/v) TG and 2.0% (v/v) with 2500 mg/L 6-AN by 12.7% and 11.3% respectively. Both mortality and malformation were both increased by approximately (reducing both the 96-hr LC50 and 96-hr EC50) the same amount thus TI should not change appreciably.

Malformation caused by all interaction treatments did not produce new or different types of malformation. All malformations were the same type as seen in the individual control treatments of each teratogen and solvent. The differences were in the magnitude of the response and number of the malformations.

The solvents tested generally caused synergistic effects with RA or 6-AN in FETAX. Mortality exhibited the greatest response to combined treatments while malformation was less affected and growth was unaffected. Interestingly, Malformation and growth inhibition are more sensitive endpoints than mortality. These results point out the need to consider the possibility of interactions for each endpoint separately and the necessity of using minimal solvent concentration. These interactions would probably cause problems with all *in vitro* aquatic bioassays. Therefore other methods of solubilizing insoluble material needs investigation.

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ASSESSING THE EFFICACY OF AN AROCLOR 1254-INDUCED
EXOGENOUS METABOLIC ACTIVATION SYSTEM FOR
FETAX

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Douglas J. Fort¹, James R. Rayburn, Donna J. DeYoung,
and John A. Bantle
Department of Zoology, Oklahoma State University
Stillwater, OK 74078

ABSTRACT

The developmental toxicity of N-nitrosodimethylamine (NDMA) and trichloroethylene (TCE) was assessed with Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Late Xenopus laevis blastulae were exposed to NDMA and TCE for 96-h in two separate static-renewal tests with and without the presence of two differently induced exogenous metabolic activation systems (MAS). The MAS consisted of Aroclor 1254-induced (Aroclor 1254 MAS) and a post-isolation mixture (mixed MAS) of Aroclor 1254- and isoniazid-induced rat liver microsomes. Addition of the mixed MAS increased the Teratogenic Index [TI=LC50/EC50 (malformation)] of NDMA and TCE nearly 2.1- and 1.7-fold, respectively. Inclusion of the Aroclor 1254 MAS did not alter the developmental toxicity of either compound. Based on TI values, embryo growth, and types and severity of induced malformations, both NDMA and TCE were developmentally toxic. Use of post-microsome isolation mixtures from differentially induced rat livers increased the efficacy of the exogenous MAS.

INTRODUCTION

Aroclor 1254 is an effective broad-spectrum
inducer of cytochrome P-450 (P-450) isozymes.¹ Thus,

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To whom correspondence should be addressed at Stover & Assoc., Inc., P.O. Box 2056, Stillwater, Oklahoma 74076

Aroclor 1254-induced liver microsomes are capable of metabolizing a diverse array of xenobiotics. Several ²⁻⁴ in vitro assay systems effectively utilize Aroclor 1254-induced exogenous metabolic activation systems (MAS), including the Frog Embryo Teratogenesis Assay ⁵ Xenopus (FETAX). However, decreased levels of putative P-450 isozymes including isoniazid-inducible P-450j have been found following administration of Aroclor 1254. ^{6,7} Thus, an Aroclor 1254-induced MAS may prove to be counter-productive for compounds metabolized by repressed isozymes.

FETAX is a whole-embryo, static-renewal bioassay designed to detect potential developmental toxicants in the environment and the workplace. ⁸ To increase predictability of FETAX, an exogenous MAS consisting of Aroclor 1254-induced rat liver microsomes was ⁵ ⁹⁻¹¹ developed and evaluated.

In this report, we evaluate the efficacy of an Aroclor 1254-induced MAS and a post-isolation mixture of microsomes induced by Aroclor 1254 and isoniazid for FETAX by assessing the developmental toxicity of two compounds thought to be metabolized by P-450j, the procarcinogenic alkylating agent N-nitrosodimethylamine (NDMA) and trichloroethylene (TCE).

MATERIALS AND METHODS

Microsome Isolation

Rat liver microsomes were isolated as described previously.⁵ Adult male Sprague-Dawley rats (150 g.) were treated with either Aroclor 1254 or isoniazid. Aroclor 1254 (500 mg/Kg) in corn oil was injected i.p.¹² 5 d prior to microsome preparation. Isoniazid (0.1% w/v, pH 7.4) was administered via drinking water for 10 consecutive d prior to microsome isolation.¹² Rats were fasted 24 h prior to microsome isolation.

Protein content was determined by the method of Bradford (BioRad, Richmond, CA).¹³ Cytochrome P-450 activity was inferred by the measurement of formaldehyde generated from the N-demethylation of aminopyrine and NDMA¹⁴ under standard assay conditions.^{6,15} Units are expressed as μ M formaldehyde generated/min.

Post-microsome isolation mixtures of Aroclor 1254-induced and INH-induced microsomes (mixed MAS) were prepared by aliquotting equal activities of aminopyrine N-demethylase (Aroclor 1254-induced preparation) and NDMA (isoniazid-induced preparation) into cryovials and snap freezing in liquid nitrogen.¹⁶ Several aliquots of microsomes were chemically reduced with dithionite and pretreated with carbon monoxide (CO-MAS) to selectively inactivate P-450 activity.¹⁷

Animal Care and Breeding

Xenopus adult care, breeding, and embryo

collection were performed as described by Dawson and
18
Bantle.

FETAX Protocol

For experiments conducted without the MAS, groups of 20 embryos were placed in 60 mm covered plastic Petri dishes with varying concentrations of the appropriate test compound. NDMA (Sigma, St. Louis, MO) and TCE (Aldrich, Milwaukee, WI) were dissolved in
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appropriate volumes of FETAX solution. For each compound, 12-18 concentrations were tested with replicates. Four separate dishes of 20 embryos were exposed to FETAX solution alone and designated FETAX solution controls. Each treatment dish contained a total of 3 ml of solution.

Tests with the MAS were also conducted in duplicate with 20 embryos per replicate concentration. Each Arcelor 1254 MAS activated treatment received 0.04
5
U/dish of aminopyrine N-demethylase activity. Mixed MAS activated treatments received 0.04 U of aminopyrine N-demethylase and NDMA N-demethylase activity per dish. Each activated treatment received an NADPH generating system and a penicillin-streptomycin mixture to control
5
bacterial growth. Controls including FETAX solution, each MAS, CO-MAS + toxicant (negative control),

7,9-
cyclophosphamide (FETAX reference MAS proteratogen
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) and acetylhydrazide (additional mixed MAS positive
7
control), and unactivated toxicant were tested
concurrently with each experiment.

For each compound, one range-finding and two
definitive dose-response assays were conducted with and
without the MAS. The pH of all stock solutions was 7.0.
Embryos were cultured at 23 ± 1 ° C. All solutions were
renewed every 24 h. Dead embryos were removed at this
time. Following 96-h of exposure, surviving embryos
were fixed in 0.7% formalin (pH 7.0). The number of
live malformed larvae and the stage of development
19
were ascertained using a dissecting microscope.

Data Analysis

96-h median lethal concentrations (LC50) and the
concentrations inducing gross terata in 50% of the
surviving larvae (EC50) along with respective 95%
confidence limits were determined using Litchfield-
20
Wilcoxon probit analysis. A Teratogenic Index
($TI = LC50/EC50$) was used as a method of assessing
5,8-11,21
teratogenic potential. Head-tail length of
surviving embryos was measured as an index of embryo
growth. Minimum concentrations to inhibit growth
(MCIG) were determined with the t-Test ($p < 0.05$).

RESULTS

In this study, FETAX solution control embryo

mortality and malformation rates were <2.0% and <5%, respectively. Mortality and malformation rates for each MAS control were <4% and <9%, respectively. Acceptable rates of FETAX solution and MAS control mortality and malformation are generally ≤10%. No less than 86% of those embryos subjected to 4.0 mg/ml activated cyclophosphamide and either MAS died. Survivors of the previous treatments were severely deformed. Exposure of embryos to 3.0 mg/ml activated acetylhydrazide and the mixed MAS produced mortality and malformation rates >43% and 100%, respectively.

Results from tests performed with NDMA are presented in Table 1. Malformations induced by unactivated and Aroclor 1254 MAS activated NDMA included miscoiling of the gut at concentrations >1.6 mg/ml. Concentrations >2.0 mg/ml produced muscular kinking and lateral body flexure. Visceral edema was observed in concentrations >3.0 mg/ml. Mixed MAS activated NDMA concentrations >0.1 mg/ml produced miscoiling of the gut and microphthalmia. Exposure to concentrations >0.5 mg/ml caused blistering of the dorsal fin, rupturing of the pigment vessicle of the eye, and skeletal kinking. Severe heart malformations were also noted at concentrations >1.0 mg/ml.

Results from tests performed with TCE are shown in Table 2. Terata induced by exposure to unactivated TCE

Table 1. Developmental toxicity of N-nitrosodimethylamine (NDMA) in FETAX.

| Endpoint | Trial | <u>Treatment</u> | | |
|---------------|-------|------------------|------------------|------------------|
| | | Unactivated | Aroclor 1254 MAS | Mixed MAS |
| LC50 (mg/ml) | 1 | 3.5 (3.3-3.7) | 2.6 (2.5-2.7) | 1.6 (1.5-1.7) |
| | 2 | 3.2 (3.1-3.3) | 2.8 (2.7-3.0) | 1.4 (1.1-1.6) |
| EC50 (mg/ml) | 1 | 2.3 (2.2-2.4) | 1.9 (1.8-2.1) | 0.6 (0.3-0.9) |
| | 2 | 2.3 (2.2-2.5) | 2.1 (2.0-2.2) | 0.4 (0.2-0.7) |
| TI | 1 | 1.5 | 1.4 | 2.7 |
| | 2 | 1.4 | 1.3 | 3.5 |
| MCIG (mg/ml) | 1 | 1.5 | 1.0 | 0.3 |
| | 2 | 1.2 | NA | 0.1 |
| MCIG (3 LC50) | 1 | 42.8 | 38.5 | 18.8 |
| | 2 | 37.5 | NA | 7.1 |

1 Represents the results of two definitive dose-response experiments with and without each of the metabolic activation systems (MAS). Median lethal (LC50) and median teratogenic (EC50) concentrations were determined by Litchfield-Wilcoxon probit analysis with 95% confidence intervals in parenthesis. Teratogenic indices were calculated by dividing the 96-h LC50 by the 96-h EC50 (malformation). Minimum concentrations to inhibit growth (MCIG) were determined by the t-test ($p < 0.05$). NA - Not available due to concentration range tested.

Table 2. Developmental toxicity of trichloroethylene (TCE) in FETAX.¹

| Endpoint | Trial | <u>Treatment</u> | | |
|---------------|-------|------------------------|------------------------|------------------------|
| | | Unactivated | Aroclor 1254 MAS | Mixed MAS |
| LC50 (ug/ml) | 1 | 425.0 (375.0-473.0) | 423.0 (390.0-453.0) | 249.0 (219.0-284.0) |
| | 2 | 443.0 (382.0-514.0) | 397.0 (375.0-439.0) | 204.0 (147.0-256.0) |
| EC50 (ug/ml) | 1 | 34.0 (20.0-55.0) | 45.0 (41.0-50.0) | 13.0 (12.0-16.0) |
| | 2 | 37.0 (33.0-43.0) | 27.0 (21.0-32.0) | 9.0 (3.0-13.0) |
| TI | 1 | 12.4 | 9.4 | 19.2 |
| | 2 | 12.0 | 14.7 | 22.7 |
| MCIG (ug/ml) | 1 | NA | 29.0 | 11.0 |
| | 2 | 29.0 | 37.0 | 7.0 |
| MCIG (% LC50) | 1 | NA | 6.9 | 4.4 |
| | 2 | 6.5 | 9.3 | 3.4 |

¹ Represents the results of two definitive dose-response experiments with and without each of the metabolic activation systems (MAS). Median lethal (LC50) and median teratogenic (EC50) concentrations were determined by Litchfield-Wilcoxon probit analysis with 95% confidence intervals in parenthesis. Teratogenic indices were calculated by dividing the 96-h LC50 by the 96-h EC50 (malformation). Minimum concentrations to inhibit growth (MCIG) were determined by the t-test (p<0.05). NA - Not available due to the concentration range tested.

and Aroclor 1254-activated concentrations >15 ug/ml were gut miscoiling and microphthalmia. Muscular kinking and incomplete development of the mouth were observed in concentrations >40 ug/ml. Concentrations exceeding 300 ug/ml also caused severe hypognathia. Mixed MAS-activated TCE concentrations >5 ug/ml produced miscoiling of the gut and craniofacial defects. Skeletal kinking, mal-development of the heart, and hydroencephaly were noted in concentrations >15 ug/ml. Concentrations >30 ug/ml also induced blistering of the dorsal fin.

DISCUSSION

Results from this study suggest that the inclusion of the mixed MAS increased the developmental toxicity of both NDMA and TCE. However, the Aroclor 1254-induced MAS itself had virtually no effect on the developmental toxicity of either compound. In FETAX, assessment of teratogenic potential is based on TI values, embryo growth, and types and severity of induced malformations. Typically, TI values <1.5 indicate low teratogenic potential, whereas higher values signify an increase in the potential teratogenic hazard. However, embryo growth and types and severity of induced anomalies are also evaluated since some compounds with TI values <1.5 cause severe malformations of major organ systems. Such compounds

may still pose a developmental hazard, possibly as embryotoxins. Compounds posing a high developmental hazard in FETAX generally inhibit growth at concentrations ²¹ <30% of the LC50.

Based on these criteria, both unactivated and metabolically activated NDMA and TCE have the potential to be teratogenic. However, only addition of the mixed MAS enhanced the teratogenic potential of NDMA and TCE.

Although the carcinogenic ^{22,23} , and to some extent, blastomogenic ^{24,25} effects of NDMA have been thoroughly studied in mammals, only few reports have described teratogenic effects. ²⁶ Bochart et al. found acetoxymethyl-nitrosomethylamine (AcO-NDMA), an acetate ester conjugate of the presumed reactive intermediate of NDMA, induced malformations in NMRI mice following administration on days 11 and 12 of gestation. Several cell culture systems ²⁷⁻²⁹ have determined NDMA to have teratogenic potential. In addition, ethanol administration to pregnant rats subjected to NDMA significantly increased the rate of fetal micronuclei ³⁰ formation in rats. Thus, interactions between P-450j inducing agents, such as, ethanol, pyrazole, and INH, could also potentiate the developmental toxicity of NDMA in mammals. The increased developmental toxicity of activated NDMA may be due to direct exposure to DNA alkylating metabolites generated by the mixed MAS.

These results are consistent with the current hypothesis that the teratogenicity of alkylating agents is closely correlated to the rate of DNA alkylation in embryonic cells.²⁶ To this point, all of the alkylating agents assayed with FETAX, including CP⁵ and NDMA which required exogenous bioactivation, have significant teratogenic potential.

Several studies evaluating the effect of maternally inhaled TCE on rat³¹⁻³³, mouse^{31,32}, and rabbit³³ fetuses have found little evidence to support a teratogenic or fetotoxic effect. However, more recent investigations suggest inhaled TCE in rats may cause a developmental delay resulting in abnormal development of the skeleton and soft tissues.^{34,35} Fetotoxicity has also been reported following oral administration in the rat.³⁶ Interestingly, TCE produced a variety of anomalies in an avian model system.^{37,38} Several of the TCE-induced malformations including cardiac effects observed in the avian model are similar to those recorded with Xenopus. Differences in the ability of TCE to induce terata in mammals, birds, and frog embryos may be the result of differences in embryonic susceptibility across the phylogenetic range, route of exposure, as well as, pharmacological nature of the test system. The majority of mammalian studies utilized pulmonary

exposure, whereas, the avian system and FETAX provide direct embryonic exposure to toxicants. Positive results in FETAX may, in fact, be due to highly embryotoxic intermediates or metabolites of TCE. The formation of a reactive epoxide intermediate (TCE-oxide) has been identified in rat, rabbit, and mouse. ³⁹ Developmentally toxic parent compounds, short-lived intermediates and metabolites exposed directly to Xenopus embryos may actually be retained in the mother in mammalian systems.

In order to evaluate human developmental toxicants, in vitro test systems must possess the ability to metabolize xenobiotics. Several compounds besides NDMA, TCE, and isoniazid have been shown to be metabolized by Aroclor-repressible P-450j including pyrazole, imidazole, acetone, and ethanol. ⁴⁰ Thus, by using mixtures of microsomes induced by a broad spectrum of P-450 inducing agents, we may increase the the overall efficacy of the MAS for FETAX.

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Altered Developmental Toxicity Caused by Three Carrier Solvents.

J.R. Rayburn¹, D.J. Fort², D.J. DeYoung¹, R. McNew³, and J.A. Bantle^{1*}

¹Department of Zoology, Oklahoma State University, Stillwater OK, 74078, USA. ²Stover & Assoc. Inc., P.O. Box 2056, Stillwater OK 74076. ³Agricultural Statistics Laboratory, University of Arkansas, Fayetteville, AR 72701, USA.

*Author to whom enquiries regarding the work should be directed.

Altered Developmental Toxicity Caused by Three Carrier Solvents.

J.R. Rayburn¹, D.J. Fort¹, D.J. DeYoung¹, R. McNew², and J.A. Bantle¹

ABSTRACT

Many aquatic bioassays rely on chemical solvents to solubilize water-insoluble test materials. Interactions between solvents and test materials can lead to false positive or negative results. For this reason, tests for interactions between solvents and test materials were performed. The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) was chosen because of its capacity to assess three different endpoints; mortality, malformation, and embryo growth. Three solvents; dimethylsulfoxide (DMSO), acetone, and triethylene glycol (TG) were tested with two teratogens; methyl-mercury chloride (MMC) and trichloroethylene (TCE). DMSO potentiated the lethal effect of both teratogens but did not significantly alter the rate of malformation. Acetone increased mortality for both teratogens, but only increased MMC malformation greater than additive effects. TG only increased mortality and malformation with TCE. There were additive effects for growth for all solvents with the teratogens. The carrier solvents caused interactions even at their No Observable Effect Concentration (NOEC). Therefore, choice of carrier solvent should be made with caution. This study shows that different results can occur depending on the solvent used and that a difference in one endpoint does not necessarily change the other endpoints.

KEYWORDS:

Xenopus; FETAX; dimethylsulfoxide; acetone; triethylene glycol; methylmercury chloride; trichloroethylene; carrier solvents; interactions; synergism.

INTRODUCTION

The large number of water-insoluble chemicals requiring toxicity testing necessitates the development, validation and use of chemical cosolvents. Carrier solvents (cosolvents), such as dimethylsulfoxide (DMSO), acetone, and triethylene glycol (TG), are commonly used to solubilize hydrophobic compounds.¹ However, the use of solvents with in vitro bioassays may alter the developmental toxicity of test materials. Solvents interact with other compounds to change rates of reactions, membrane potentials, mutagenic activity, and many other cell processes.²⁻⁵ Using microbial assays, Stratton has shown that solvents alter the toxicity of pesticides.⁶ For this reason, solvent-compound interaction studies were performed to determine if the developmental toxicity of test materials was altered.

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), described by Dumont et al., is a 96-hr bioassay which determines relative teratogenic hazard.⁷ Several labs have evaluated compounds as well as environmental mixtures with FETAX.⁷⁻¹² An exogenous metabolic activation system has been developed and evaluated for FETAX.¹³⁻¹⁵ The purpose of these experiments was to determine whether carrier solvents interacted with the teratogens methylmercury chloride and trichloroethylene to affect survival, development and growth of *Xenopus* embryos.

EXPERIMENTAL

Chemicals

Solvents were dimethylsulfoxide (DMSO) (CAS# 67-68-5; Sigma Chemical Co., St. Louis, MO) acetone (CAS# 67-64-1; Fisher Scientific, Houston, TX), and triethylene glycol (TG) (CAS# 112-27-6; Aldrich Chemical Co., Milwaukee, WI). Teratogens were methylmercury chloride (MMC) (CAS# 115-09-3; Pfaltz & Bauer Inc., Waterbury, CT) and trichloroethylene (TCE) (CAS# 79-01-6; Aldrich Chemical Co.).

Assay procedure

Animal care and breeding were performed according to Bantle et al. (1989).¹⁶ FETAX solution, a reconstituted water medium, was used as the diluent for all experiments.¹⁷ For each concentration-response test, two groups of 25 embryos each were placed in 60 X 15 mm glass Petri dishes containing a total of 10 ml of solution. Four groups of 25 embryos were exposed to FETAX solution and used as controls for each test. Because of the possible binding of methylmercury to glass, the concentration-response experiments were performed in 60 X 15 mm plastic Petri dishes containing 20 embryos and a total of 8 ml of solution. Each experiment followed standard methods of test operation and embryo evaluation.^{16,18} Stock concentrations of MMC were also determined using the cold vapor method for determining mercury concentration.¹⁹

One range and three definitive experiments were performed to determine the 96-hr LC50, 96-hr EC50 (malformation), Teratogenic Index (TI) (96-hr LC50/ 96-hr EC50) and Minimum Concentration to Inhibit

Growth (MCIG) for three solvents and two teratogens. The 96-hr, LC25 and LC50, and 96-hr, EC25 and EC50 (malformation) were determined using Litchfield-Wilcoxon probit analysis.²⁰ Dunnett's test was used to determine the No Observable Effect Concentrations (NOEC) for malformation and mortality. The MCIG was determined by comparing head-tail lengths between control and experimental groups using the t-test for grouped observations. These data were used in determining test concentrations for the solvent-compound interaction study. Malformations were determined using a dissecting microscope to observe deviations from normal development.²¹ Typical examples of malformations are reduced head compared with rest of embryo, reduced eye, mouth, misshapen head, eye, heart, mouth, tail fin, notocord, etc. The 96-hr embryo is transparent and most internal organs can be seen clearly. Stunting (growth inhibition) is not considered a malformation. Growth is assessed by measuring embryo head-tail lengths.

Interaction Study

Two concentrations for each solvent and test material were selected. The two solvent concentrations were the NOEC and 96-hr EC25. The NOEC is the highest possible cosolvent concentration that can be used in FETAX. The 96-hr EC25 was chosen in order to positively identify any interactions that might be taking place that were not observable at lower concentrations. MMC and TCE concentrations were the 96-hr EC25 and 96-hr LC25. These concentrations allowed examination of effects on both malformation and mortality. Both MMC and TCE were soluble at these

concentrations without the solvents. The interaction experiments contained individual treatments for all concentrations and the interaction treatments for one solvent and one teratogen per experiment. The nine treatments were: 1) FETAX solution controls, 2) EC25 of teratogen, 3) LC25 of teratogen, 4) NOEC level of solvent, 5) EC25 of solvent, 6) EC25 of teratogen & NOEC of solvent, 7) EC25 of teratogen & EC25 of solvent, 8) LC25 of teratogen & NOEC of solvent, and 9) LC25 of teratogen & EC25 of solvent. All interaction experiments contained four replicates of 25 embryos each per treatment with the exception of one MMC-DMSO interaction study which contained 22 embryos per treatment. Results of solvent-teratogen interaction studies represent three pooled experiments using three different breeding pairs. Every 24 hrs, dead embryos were removed and solutions changed. After 96 hrs of exposure, embryos were anesthetized with 3-Aminobenzoic Acid Ethyl Ester (MS-222) (CAS# E1-052-1; Sigma) and the number of malformed larvae recorded. Larvae were then killed and fixed with 3.0% (w/v) formalin and head-tail length (growth) measured.

ANOVA was used to determine differences from theoretical additive values for mortality, malformation and growth.²² Values were determined by partitioning the sum of squares for the interactions on mortality and malformation using the statistical software SYSTAT.²³ Growth interactions were determined in the same manner with the Statistical Analysis System (SAS). Effects on the TI were inferred from shifts of the mortality and malformation curves. In the case of the LC25 of the teratogens,

100% malformations were obtained without solvent addition. Therefore, only a decrease in malformation could be observed and not an increase. For these experiments, decreases in malformation were not observed.

RESULTS

PRELIMINARY EXPERIMENTS

Carrier Solvent Results

Previous work indicated that all solvents caused effects at 2.0% (v/v) concentrations. Acetone produced the highest TI value of the solvents followed by DMSO and then TG.²⁴ NOEC levels for DMSO, acetone, and TG for any endpoint were 1.0% (v/v), 0.9% (v/v), and 1.7% (v/v), respectively. The 96-hr EC25 (malformation) for DMSO, acetone and TG were 1.2% (v/v), 1.0% (v/v), and 2.0% (v/v), respectively.

Developmental Toxicity of the Teratogens

Both methylmercury chloride and trichloroethylene proved to have teratogenic potential in FETAX. The lower TI obtained for MMC indicated that it was less teratogenic than TCE (Table 1). The 96-hr EC25 and LC25 for MMC were 0.015 mg/L and 0.088 mg/L. For TCE, they were 0.002% (v/v) and 0.035% (v/v). It was not possible to determine MCIG or confidence limits for the first TCE test. However, acceptable confidence limits were obtained in all other tests. The data from these experiments was used to predict the EC25 and LC25 for the teratogens. The estimation of the EC25 for the three independent experiments for TCE ranged from 19.5% to 27.5% and the estimation of the LC25 ranged from 25.7% to 38.3% (Tables 5-7). Therefore, the estimates of EC25 and LC25 were well within acceptable limits. The mean MCIG for MMC was 0.038 mg/L and 0.02% (v/v) for TCE.

INTERACTION EFFECTS CAUSED BY SOLVENTS

Control Results

Tables 2 through 7 show the pooled percentage results for all experiments. Control mortality and malformation was equal to or below 10% for all but three experiments and was never greater than 12%. Although the control malformation and mortality varied from experiment to experiment, final results were not affected because ANOVA takes into account control responses. Table 2 - Table 4 shows that mortality due to 0.088 mg/L MMC ranged from 18.0% to 41.7%. Because of this variability, values were kept discrete for each solvent-teratogen interaction. These are the percentages used to calculate the theoretical additive values. The percentages are the 12 replicate values averaged together, and the standard error is the error of these 12 means.

Methylmercury Chloride Interactions

MMC combined with DMSO caused significant ($p = 0.05$) interaction only at 1.2% (v/v) DMSO and 0.088 mg/L MMC level (Figure 1). Mortality was increased by 18% (6-35%; 95% confidence interval). In all cases, the percent change refers to an increase or decrease compared to the theoretical additive value. DMSO increased the mortality which should decrease the 96-hr LC50 and did not significantly increase malformation. Therefore, the 96-hr EC50(malformation) should remain the same with DMSO. This would reduce the TI of MMC. One experiment contained 22 embryos per replicate due to a shortage of embryos, and is the reason

the total N per treatment is different. All experiments showed similar results.

When MMC was tested with acetone there were significant interactions observed for both mortality and malformation (Figure 1). MMC at 0.015 mg/L with 0.9% (v/v) and 1.0% (v/v) acetone increased malformation by 16.9% (10.9-23.8%; 95% confidence interval) and 28.6% (21.0-36.9%; 95% confidence interval), respectively. MMC at 0.088 mg/L with 1.0% (v/v) acetone increased the mortality by 23.7% (10.8-40%; 95% confidence interval). Because the synergistic responses for malformation and mortality were approximately the same, acetone would change the 96-hr LC50 and 96-hr EC50 (malformation) the same. The TI, therefore, should not change.

MMC combined with TG showed no significant interactions on mortality or malformation (Figure 1). TG, therefore, would not change the TI of MMC.

The TI would be expected to change for MMC only with DMSO. With acetone, the interaction is an equal increase in mortality and malformation over the expected additive value. This would increase the sensitivity of the embryos to MMC when combined with acetone. Finally, when MMC was tested with TG neither mortality or malformation were changed. MMC growth was not changed from additive effects for any of the solvents.

Trichloroethylene Interactions

Trichloroethylene combined with DMSO had significant ($p = 0.001$) additive effects on mortality (Figure 2). Mortality for 0.035% (v/v) TCE was increased over theoretical additive values for DMSO at 1.0% (v/v) and 1.2% (v/v) by 42.7% (21.7-67.7%; 95% confidence interval) and 45.3% (23.7-70.6%; 95% confidence interval), respectively. However, malformation was not changed significantly from theoretical additive values. The increase in mortality resulted in a decreased 96-hr LC50. Because malformation was unaffected, the TI would be lowered.

TCE combined with acetone significantly ($p = 0.05$) increased mortality (Figure 2). The rate of mortality caused by TCE at 0.035% (v/v) was increased with acetone at 0.9% (v/v) and 1.0% (v/v) by 16.7% (6.4-26.7; 95% confidence interval) and 24% (13-37.5%; 95% confidence interval), respectively. The rate of TCE-induced malformation at 0.002% (v/v) was not significantly different from theoretical additive values. An increase in mortality for acetone and no change in malformation from theoretical values would again cause a reduction of the TI.

TCE combined with TG caused significant ($p = 0.05$) interaction for both mortality and malformation (Figure 2). The rate of TCE-induced mortality caused by 0.035% (v/v) TCE with 1.7% (v/v) and 2.0% (v/v) TG was increased over theoretical additive values by 15% (6.2-26.9%; 95% confidence interval) and 20.33% (9.97-33.4%; 95% confidence interval), respectively. The malformation caused by 0.002% (v/v) TCE and 2.0% (v/v) TG was significantly increased by 17.2% (10.9-24.6%; 95% confi-

dence interval). Because both malformation and mortality for TG showed an increase of approximately the same magnitude, the TI would not be expected to change.

Trichloroethylene with DMSO and TCE with acetone caused an increase in mortality and did not change malformation significantly. TCE with TG, however, increased both mortality and malformation.

DISCUSSION

The Teratogenic Index (TI) is the ratio of the 96-hr LC50/96-hr EC50 (malformation) and represents the separation between the mortality and malformation curves. It would be expected that for compounds to induce malformations without causing mortality that the 96-hr EC50 (malformation) should be less than the 96-hr LC50. This is not, however, the only way to classify compounds because the number of malformations do not give information on the types or severity of these malformations. The separation between the two curves is considered to be sufficient to pose significant teratogenic hazard when the $TI > 1.5$.^{16,18} If a synergistic or antagonistic response altered a TI, false conclusions regarding teratogenic hazard could result.²⁵

MMC is known to cause cleft palate in mice.²⁶ MMC is also known to affect development of several fetal systems.²⁷⁻²⁹ TCE is known to induce strand breaks in DNA in the rat and mouse liver in vivo and to be weakly mutagenic.³⁰⁻³¹ TCE causes abnormal development of chick embryos.³²⁻³³ However, TCE does not show strong teratogenic effects in the rat inhalation studies but did produce skeletal anomalies.³⁴ Route of exposure is important when comparing relative toxicity, teratogenicity. TCE seems to show wide species variation in response. These sources support that MMC and, perhaps, TCE should be teratogenic in FETAX.

The interaction results show that the carrier solvents do interact with teratogens. Of the three indicators measured, mortality and malformation were potentiated, while growth was not affected. Typically, growth and

malformation are the more sensitive endpoints. However, more interactions were discovered with mortality. This shows that a change in one endpoint does not necessarily result in a change in the others. This study also shows that the choice of carrier solvents is critical because the different carrier solvents caused different interactions. For example, only acetone caused effects at the NOEC with MMC. However, all solvents caused effects when combined with TCE at the NOEC. While TG had no observed effects with MMC, both malformation and mortality were significantly changed with TCE.

Malformations caused by all interaction treatments did not produce new or different types of malformation. All malformations were the same type as seen with individual control treatments for each teratogen and solvent. Differences were in the magnitude of the response and the number of malformations. Acetone has previously been shown to change mutagenic potential of N-Methyl-N-nitrosourea and to interact on membrane integrity.^{5,35} DMSO has been shown to interact with secalon-ic acid D to alter teratogenicity in mice.³⁶ These papers support the results of this study where interactions were found between MMC and TCE, and the three solvents.

Carrier solvents, although sometimes necessary, need to be used with caution at the lowest solvent concentrations possible. If possible, several different carrier solvents should be used separately to determine if the results are consistent.³⁷ Fewer effects were seen at the NOEC than at the 96-hr EC50 for the solvents. Also interesting, is the finding that

MMC was the least teratogenic and had the fewest interactions, while TCE, with a higher TI, had more interactions. These results are also supported by Rayburn et al.²⁴, who studied the interactions of the three solvents, DMSO, acetone and TG with t-retinoic acid and 6-aminonicotinamide. They documented more interactions with mortality than malformation, observed no interactions on growth and the stronger teratogen, 6-aminonicotinamide, showed more interactions than t-retinoic acid.

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Table 1. FETAX 96-hr LC50, 96-hr EC50(malformation), Teratogenic Index (TI)^a, and Minimum Concentration to Inhibit Growth (MCIG) for Methylmercury Chloride (MMC) and Trichloroethylene (TCE).

| Compound | | 96-hr LC50 | 96hr EC50 | TI | MCIG |
|--------------------|---|----------------------------------|---------------------|------|-------|
| <u>Trial</u> | | | | | |
| MMC ^b | 1 | 0.083(0.080-0.087 ^c) | 0.024(0.021-0.028) | 3.4 | 0.036 |
| | 2 | 0.094(0.088-0.100) | 0.025(0.018-0.034) | 3.7 | 0.04 |
| TCE ^{d e} | 1 | 0.024(NA) | 0.0048(0.002-0.011) | 5 | NA |
| | 2 | 0.029(0.026-0.032) | 0.0023(0.001-0.004) | 12.6 | 0.02 |

^a TI = 96-hr LC50/96-hr EC50(malformation).

^b Concentrations expressed as mg/L.

^c 95% confidence limits.

^d Concentrations expressed as % (v/v).

^e Density = 1.462.

NA = Not Available.

Table 2. Effects of Dimethyl Sulfoxide (DMSO), Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|----------------------------|--------------|---------------|--------------|
| | No. | (%) | No. | (%) | (mm) |
| FETAX soln. | | | | | |
| Control | 18 | (6.3 ± 2.72 ^b) | 18 | (7.0 ± 1.42) | 9.34 ± 0.069 |
| DMSO MMC | | | | | |
| % (v/v) (mg/L) | | | | | |
| 1.0 | 23 | (8.3 ± 1.94) | 24 | (9.4 ± 1.34) | 9.16 ± 0.069 |
| 1.2 | 27 | (9.6 ± 2.43) | 75 | (29.8 ± 3.92) | 8.95 ± 0.072 |
| 0.015 | 19 | (6.7 ± 2.71) | 50 | (19.3 ± 1.65) | 9.22 ± 0.086 |
| 0.088 | 117 | (41.7 ± 8.26) | 163 | (100) | 7.55 ± 0.129 |
| 1.0 0.015 | 22 | (7.7 ± 2.33) | 65 | (25.0 ± 2.34) | 9.21 ± 0.086 |
| 1.2 0.015 | 70 | (25.0 ± 7.75) | 109 | (51.8 ± 9.18) | 8.85 ± 0.090 |
| 1.0 0.088 | 123 | (44.0 ± 9.62) | 157 | (100) | 7.88 ± 0.213 |
| 1.2 0.088 | 176 | (63.0 ± 10.33) | 104 | (100) | 7.68 ± 0.253 |

^a N for all treatments equalled 288 embryos from three separate experiments. Two experiments contained 25 embryos per dish (8 dishes), and one experiment contained 22 embryos per dish (4 dishes).

^b Standard Error of Mean.

Table 3. Effects of Acetone, Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|--------------------------------|--------------|-------------------|------------------|
| | No. | (%) | No. | (%) | (mm) |
| FETAX soln. | | | | | |
| Control | 22 | (7.3 \pm 2.08 ^b) | 33 | (11.8 \pm 2.01) | 9.47 \pm 0.069 |
| Acetone | | | | | |
| MMC | | | | | |
| % (v/v) | | | | | |
| (mg/L) | | | | | |
| 0.9 | 23 | (7.7 \pm 2.89) | 56 | (20.3 \pm 1.55) | 8.97 \pm 0.064 |
| 1.0 | 16 | (5.3 \pm 1.50) | 100 | (35.2 \pm 1.95) | 8.78 \pm 0.075 |
| 0.015 | 18 | (6.0 \pm 2.44) | 63 | (22.2 \pm 0.97) | 9.41 \pm 0.130 |
| 0.088 | 54 | (18.0 \pm 4.45) | 246 | (100) | 8.85 \pm 0.127 |
| 0.9 0.015 | 21 | (7.0 \pm 2.04) | 133 | (47.6 \pm 4.17) | 8.95 \pm 0.098 |
| 1.0 0.015 | 32 | (10.7 \pm 2.76) | 199 | (74.2 \pm 5.59) | 8.70 \pm 0.104 |
| 0.9 0.088 | 89 | (29.7 \pm 7.65) | 211 | (100) | 7.81 \pm 0.135 |
| 1.0 0.088 | 119 | (39.7 \pm 10.16) | 181 | (100) | 7.71 \pm 0.107 |

^a N for all treatments equalled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 4. Effects of Triethylene Glycol(TG), Methylmercury Chloride (MMC) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length (mm) |
|------------------------|-----------|-----------------------------|--------------|---------------|---------------------|
| | No. | (%) | No. | (%) | |
| FETAX soln. Control | 30 | (10.0 ± 2.00 ^b) | 25 | (9.4 ± 1.47) | 9.27 ± 0.084 |
| TG MMC | | | | | |
| % (v/v) (mg/L) | | | | | |
| 1.7 | 15 | (5.0 ± 0.87) | 35 | (12.3 ± 1.41) | 9.14 ± 0.087 |
| 2.0 | 21 | (7.0 ± 2.04) | 103 | (36.8 ± 3.48) | 8.88 ± 0.087 |
| 0.015 | 24 | (8.0 ± 2.46) | 59 | (21.5 ± 0.99) | 8.99 ± 0.092 |
| 0.088 | 81 | (27.0 ± 10.00) | 219 | (100) | 8.00 ± 0.187 |
| 1.7 0.015 | 17 | (5.7 ± 1.59) | 93 | (32.7 ± 2.80) | 9.33 ± 0.066 |
| 2.0 0.015 | 19 | (6.3 ± 3.21) | 160 | (57.1 ± 5.80) | 9.19 ± 0.118 |
| 1.7 0.088 | 60 | (20.0 ± 6.73) | 240 | (100) | 8.06 ± 0.269 |
| 2.0 0.088 | 51 | (17.0 ± 4.15) | 249 | (100) | 8.07 ± 0.199 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos each.

^b Standard Error of Mean.

Table 5. Effects of Dimethyl Sulfoxide(DMSO), Trichloroethylene (TCE) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|----------------------------|--------------|---------------|--------------|
| X(v/v) | No. | (%) | No. | (%) | (mm) |
| FETAX soln. | | | | | |
| Control | 22 | (7.3 ± 3.07 ^b) | 25 | (9.0 ± 1.72) | 9.35 ± 0.101 |
| DMSO TCE | | | | | |
| 1.0 | 18 | (6.0 ± 2.63) | 31 | (11.0 ± 1.35) | 9.14 ± 0.072 |
| 1.2 | 31 | (10.3 ± 4.07) | 77 | (28.8 ± 2.42) | 8.92 ± 0.124 |
| 0.002 | 21 | (7.0 ± 6.29) | 58 | (20.9 ± 1.12) | 9.40 ± 0.118 |
| 0.035 | 115 | (38.3 ± 10.69) | 185 | (100) | 7.73 ± 0.115 |
| 1.0 0.002 | 16 | (5.3 ± 1.80) | 81 | (28.7 ± 2.04) | 9.14 ± 0.089 |
| 1.2 0.002 | 42 | (14.0 ± 4.13) | 118 | (45.9 ± 1.78) | 8.92 ± 0.109 |
| 1.0 0.035 | 239 | (79.7 ± 9.24) | 61 | (100) | 7.55 ± 0.219 |
| 1.2 0.035 | 260 | (86.7 ± 6.44) | 40 | (100) | 7.08 ± 0.112 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Table 6. Effects of Acetone, Trichloroethylene (TCE) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|----------------------------|--------------|---------------|--------------|
| %(v/v) | No. | (%) | No. | (%) | (mm) |
| FETAX soln. Control | 17 | (5.7 ± 2.00 ^b) | 15 | (5.15 ± 1.44) | 9.36 ± 0.069 |
| Acetone TCE | | | | | |
| 0.9 | 37 | (12.3 ± 4.52) | 47 | (17.9 ± 1.49) | 9.01 ± 0.066 |
| 1.0 | 19 | (6.3 ± 2.12) | 124 | (44.3 ± 4.13) | 8.91 ± 0.095 |
| 0.002 | 11 | (3.7 ± 1.51) | 56 | (19.5 ± 1.26) | 9.31 ± 0.072 |
| 0.035 | 77 | (25.7 ± 7.84) | 223 | (100) | 8.21 ± 0.179 |
| 0.9 0.002 | 34 | (11.3 ± 3.30) | 111 | (41.7 ± 2.93) | 9.02 ± 0.101 |
| 1.0 0.002 | 21 | (7.0 ± 3.94) | 175 | (62.7 ± 4.41) | 8.88 ± 0.081 |
| 0.9 0.035 | 147 | (49.0 ± 7.61) | 153 | (100) | 7.69 ± 0.133 |
| 1.0 0.035 | 151 | (50.3 ± 9.33) | 149 | (100) | 7.76 ± 0.268 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Table 7. Effects of Triethylene Glycol (TG), Trichloroethylene (TCE) and their Interactions on Mortality, Malformation and Growth.

| Treatment ^a | Mortality | | Malformation | | Mean Length |
|------------------------|-----------|-----------------------------|--------------|---------------|--------------|
| %(v/v) | No. | (%) | No. | (%) | (mm) |
| FETAX soln. Control | 32 | (10.7 ± 2.80 ^b) | 29 | (10.8 ± 2.35) | 9.29 ± 0.127 |
| TG TCE | | | | | |
| 1.7 | 18 | (6.0 ± 1.87) | 63 | (22.2 ± 2.31) | 9.13 ± 0.121 |
| 2.0 | 23 | (7.7 ± 2.17) | 101 | (36.4 ± 2.41) | 8.76 ± 0.165 |
| 0.002 | 32 | (10.7 ± 2.33) | 74 | (27.5 ± 2.70) | 9.24 ± 0.129 |
| 0.035 | 99 | (33.0 ± 9.57) | 201 | (100) | 8.03 ± 0.292 |
| 1.7 0.002 | 25 | (8.3 ± 2.28) | 132 | (48.1 ± 7.05) | 8.91 ± 0.150 |
| 2.0 0.002 | 27 | (9.0 ± 2.15) | 192 | (70.3 ± 6.97) | 8.60 ± 0.182 |
| 1.7 0.035 | 130 | (43.3 ± 10.92) | 170 | (100) | 7.62 ± 0.354 |
| 2.0 0.035 | 151 | (50.3 ± 11.01) | 149 | (100) | 7.39 ± 0.387 |

^a N for all treatments equaled 300 embryos from three separate experiments. There were 12 dishes of 25 embryos per dish.

^b Standard Error of Mean.

Figure 1.

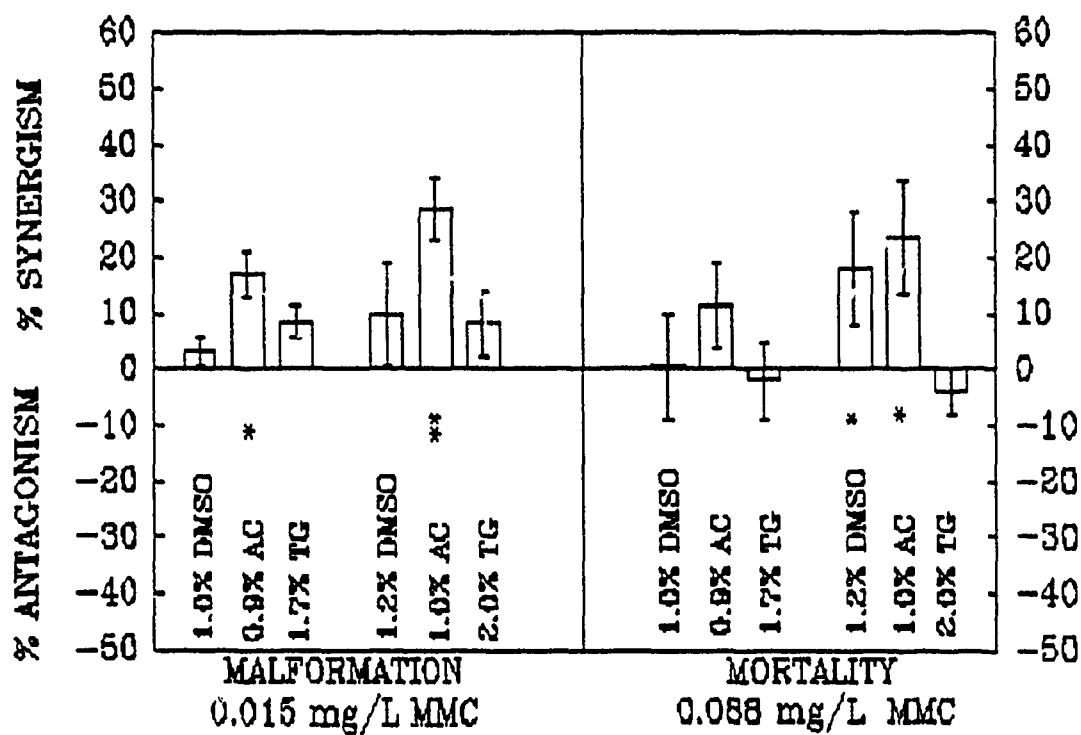


Figure 1. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for methylmercury chloride (MMC). Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments.

Figure 2

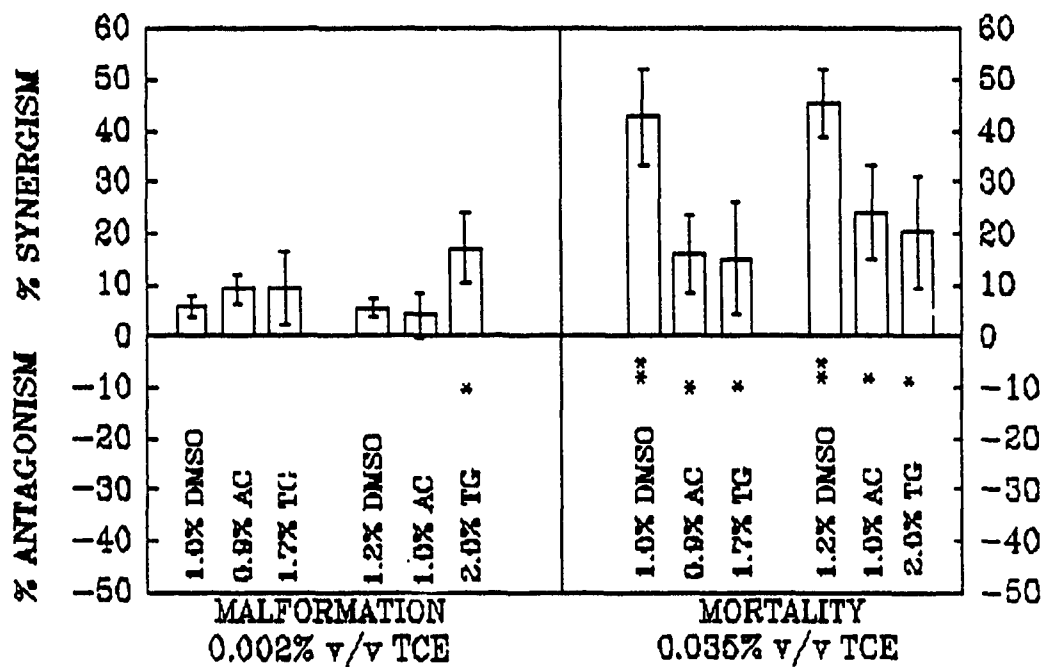


Figure 2. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints

for

trichloroethylene (TCE). Zero value represents the expected additive effect for the solvent and test com-

pound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * =

significantly different at $p=0.05$ and ** = significantly different at $p=0.001$. Solvents are Dimethyl Sulfoxide

(DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between

experiments.

ASSESSMENT OF THE DEVELOPMENTAL TOXICITY OF ASCORBIC ACID, SODIUM SELENATE, COUMARIN, SEROTONIN, AND 13-CIS RETINOIC ACID USING FETAX

Donna J. DeYoung, John A. Bantle¹, and Douglas J. Fort
Department of Zoology, Oklahoma State University,
Stillwater, OK 74078 USA

ABSTRACT

The developmental toxicity of five compounds was evaluated with the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) and the results were compared to mammalian literature. Small cell *Xenopus laevis* blastula were exposed to ascorbic acid, sodium selenate, coumarin, serotonin and 13-cis retinoic acid for 96-hr. Three separate static-renewal assays were conducted for each compound. Teratogenic potential of the test materials was determined based on Teratogenic Index values [$TI = LC50(\text{mortality}) / EC50(\text{malformation})$], types and severity of induced malformations and embryo growth. Ascorbic acid had little or no teratogenic potential. Sodium selenate and coumarin tested as having moderately positive teratogenic potential. Serotonin scored as having moderately strong teratogenic potential and 13-cis retinoic acid scored as having strong teratogenic potential. Results are consistent with mammalian data and support the use of FETAX for the screening of developmental toxicants.

INTRODUCTION

The Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) is a rapid, cost-effective alternative to mammalian teratogenesis assay systems and is helpful for identifying developmental toxicants.¹ Data may be used for prioritizing samples for further tests which currently use mammals.

FETAX is a 96 hr static-renewal assay capable of determining the developmental toxicity of pure compounds²⁻⁴ and complex mixtures.⁵⁻⁷ In addition to FETAX, a successful metabolic activation system (MAS) has been developed⁸⁻⁹, evaluated¹⁰⁻¹¹, and applied to the study of toxicological mechanisms of teratogenesis.¹¹⁻¹³

¹To whom correspondence should be addressed.

In this report, we evaluate the developmental toxicity of five compounds using FETAX and compare the results to mammalian literature.

MATERIALS AND METHODS

Animal care, frog breeding and embryo collection were performed according to Dawson and Bantle.³

Sets of 20 or 25 embryos were placed in 60-mm covered glass Petri dishes with a range of concentrations appropriate to each test compound (Sigma Chemical Co., St. Louis, MO) dissolved in FETAX solution.³ For each compound 8 to 16 concentrations were tested in duplicate. Embryos were exposed to FETAX in four separate control dishes of 20 or 25 embryos each. Treatment and control dishes contained a total of 10 ml of solution for 25 eggs exposed and 8 ml for 20 eggs exposed. Dimethyl sulfoxide was used as a solvent for coumarin at a concentration less than 1.1% v/v which has been found not to cause any adverse effects in FETAX. Because of the relative insolubility of 13-cis retinoic acid, stocks were prepared by measuring 1 mg into 1 L FETAX solution, stirring and filtering with 0.45 μ m millipore filter paper. The stock concentration was then determined by spectrophotometry with a wavelength setting of 354 nm¹⁴.

At least one range and three definitive concentration-response tests were conducted for each compound. Tests run separately by different technicians were analyzed together statistically. The pH of all compounds tested was between 7.0 and 8.0. Embryos were cultured at 24°C¹ throughout the test.

Solutions were renewed every 24 hr for a total exposure time of 96 hr and dead embryos were removed daily. At the termination of the tests, surviving embryos were anesthetized with 3-aminobenzoic acid ethyl ester (methanesulfonate salt) and fixed with 3.0% (w/v) formalin. The number of dead, number and type of malformations, and developmental stages were recorded and determined using a dissecting microscope.¹⁵

Probit analysis¹⁶ determined the 96 hr LC50 (median lethal concentration), 96 hr EC50 (concentration inducing malformations in 50% of the surviving embryos) and 95% confidence intervals for each test compound. In order to compare and assess levels of teratogenic potential 2-4,8-12, a Teratogenic Index [TI=LC50 (mortality)/EC50 (malformation)] was also determined.

Head-tail length (growth) data was collected at the end of each definitive test using an IBM-compatible computer equipped with digitizing software (Jandel Scientific, Corte Madera, CA). The Minimum Concentration to Inhibit Growth (MCIG) was calculated using the t-Test for grouped observations at the $p < 0.05$ level.

RESULTS

Final results from the definitive tests with FETAX are presented in Table 1. Representative concentration-response and growth-inhibition curves for the five compounds are illustrated in Figures 1 and 2, respectively. In this investigation the FETAX solution control mortality and malformation rates were 44 of 1300 (3.4%) and 72 of the 1256 survivors (5.7%), respectively. Control data for dimethyl sulfoxide, the solvent used in the

testing of coumarin, was 1 of 130 (0.8%) for mortality and 5 of 129 survivors (3.8%) for malformation. Acceptable rates of control mortality and malformation are generally $< 10\%$.

The most common malformation induced by ascorbic acid was failure of the gut to coil. At concentrations > 10 mg/ml loose gut coiling was common along with slight musculoskeletal kinking. At concentrations > 13 mg/ml facial, eye and brain malformations were also noted. Growth was stunted and severe malformations of the gut, musculoskeletal system, face, eye and heart occurred at concentrations > 19 mg/ml.

Sodium selenate at concentrations > 0.002 mg/ml resulted in embryos with edema and malformations of the gut, heart and face. Blistering was evident at concentrations > 0.012 mg/ml.

Coumarin induced musculoskeletal kinking, loose gut coiling and craniofacial malformations at concentrations > 0.01 mg/ml. Concentrations > 0.04 mg/ml induced craniofacial malformations consisting of a reduced head size and downward tilting of the head. Edema, eye and gut malformations occurred at concentrations > 0.07 mg/ml. The malformations mentioned above became more severe at concentrations above 0.13 mg/ml.

Serotonin caused minor malformations and stunting at most of the concentrations tested. Embryos gradually became smaller, shorter in length, less developed, with blunter nose, and looser gut coil. At concentrations > 1.0 mg/ml, microencephaly and blistering of the dorsal fin were noticed. Embryos were severely stunted at concentrations > 3.0 mg/ml.

13-cis retinoic acid caused loose gut coiling and musculoskeletal kinking at concentrations > 0.5 ng/ml. Concentrations > 2.0 ng/ml resulted in eye and brain malformations. Cyclopia, eye pigment ruptures, edema, spinal kinking and craniofacial abnormalities were induced in concentrations > 10 ng/ml.

DISCUSSION

FETAX determines teratogenic potential by comparing TI values, embryo growth, and the type and severity of induced malformations. In general, TI values < 1.5 indicate low teratogenic potential and higher values indicate an increase in the potential hazard.^{2-4,8-12} With higher TI values, the mortality and malformation dose-response curves become separated and the potential for the production of deformed embryos in the absence of lethality increases³. In this investigation, ascorbic acid, TI of 1.6, exhibited an overlapping of the curves as presented in Figure 1. It is therefore considered embryo-lethal at high concentrations and is not a potential teratogen. Sodium selenate and coumarin, with TI values of 3.0 and 3.2 respectively, represent compounds with increasing separation of the curves and potential teratogenic hazard. Serotonin and 13-cis retinoic acid, TI values of 7.6 and 12.7 respectively, exhibit wider separation of the curves, and are examples of compounds with strong teratogenic hazard. Because all chemicals are potential teratogens if administered in appropriate doses at sensitive stages of development¹⁷, it is important to consider the types and severity of terata and the concentrations at which they occur. Compounds

with TI values < 1.5 may pose a hazard to developing organisms, possibly as embryotoxins.

Developmental toxicity may also be assessed by considering the MCIG (expressed as % compound LC50)^{2-4,10}. Rates of growth inhibition (i.e. slope) and overall reduction in embryo growth vary with the severity of the teratogen. Dawson et al⁴ suggest that compounds with significant teratogenic potential generally inhibit growth at concentrations $< 30\%$ of the respective LC50 values. Ascorbic acid begins to inhibit growth at $> 50\%$ of the LC50. Sodium selenate and coumarin cause inhibition between 28 and 48% of the LC50. Serotonin and 13-cis retinoic acid cause inhibition between 10% and 16% of each particular LC50. In addition, serotonin and 13-cis retinoic acid with the highest TI values show a sharper decrease in slope compared to the other compounds. This characteristic is presented in Figure 2 by the growth-inhibition curves.

The five compounds presented here have been selected to be part of the validation process of FETAX because of the availability of mammalian literature for each¹⁸⁻¹⁹. Although FETAX results cannot be directly extrapolated to mammalian developmental toxicity tests, comparisons are beneficial in order for FETAX to be useful as a screening assay.

Ascorbic acid which tested negative in FETAX, has been tested with mice, rats and rabbits in studies following FDA Segment II guidelines. No effects were observed in rats up to 500 or 1000 mg/kg/dy²⁰⁻²¹. No effect was observed in mice up to 1000 mg/kg²¹ or in rabbits up to 500 mg/kg/dy²⁰. Frohberg et al.²¹ administered up to 1000 mg by mouth to pregnant mice and rats on days 6-15 with no adverse effects found. In fact,

ascorbic acid (ascorbate) has been found to protect against the embryolethality of N-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene (NF), and decreased the number of flexure abnormalities caused by NF in a rat whole embryo culture system²².

FETAX results for sodium selenate also agree with the majority of mammalian literature reports. As a potential teratogen it tested positive in FETAX with a TI of 3.0. Selenium occurs as selenate (Se^{6+}), selenite (Se^{4+}), elemental selenium (Se^0), and selenide (Se^{2-}). Selenium induced malformations have resulted from livestock grazing on seleniferous ranges.²³⁻²⁴ Beath et al.²⁴ reported malformations in lambs consisting of multiple cysts in eyes, microphthalmia and deformities of the extremities. Similar effects were reported with horses²⁵. However, early laboratory studies with rats and cats fed a continuous diet of selenium did not result in any malformations in the progeny²⁶. As a result of the interest in the Kesterson Reservoir and Kesterson National Wildlife Refuge, Merced County, California, in which selenium contamination was a factor, several developmental toxicity studies have emerged. Sodium selenite and selenomethionine were tested in the laboratory with mallards and malformations found included hydrocephaly, microphthalmia, lower bill and foot defects, edema and stunted growth²⁷. High rates of embryonic mortality and abnormalities were also reported in wild aquatic bird populations at Kesterson²⁸⁻²⁹. An *in situ* study was conducted using 10 species of mammals collected from Kesterson Reservoir and a low rate of abnormalities was found.³⁰ Nobunaga et al.³¹ conducted a study with mice

in which the malformation rate of sodium selenite was not significantly different from controls. Species differences to developmental toxicity caused by selenium are apparent, however FETAX results agree with the species tested other than rats and mice and further studies are needed.

Coumarin is a compound which also has conflicting reports in the mammalian literature but is generally considered to be a developmental toxin, especially for humans. Coumarin-induced abnormalities are known as the fetal warfarin syndrome³² and the most consistent malformations in humans have been described by Shaul and Hall³³ as nasal hypoplasia, stippling of the bones, ophthalmologic abnormalities, intrauterine growth retardation and developmental delay. When administered to mice, coumarin elicited a low incidence of gross fetal malformations including cleft lip and cleft palate.³⁴ A study on rabbits and mice found that coumarin adversely affected normal implantation and placentation, but no mention was made of any malformations.³⁵ In a similar study by Hirsch et al.³⁶, rabbits exposed to coumarin gave birth to stillborn fetuses with hemorrhages. One discrepancy between nonhuman and human data may be due to the fact that in man and the baboon, coumarin is metabolized to 7-hydroxycoumarin³⁷⁻³⁸ but this metabolic pathway is relatively minor in the rat. FETAX tested coumarin as a positive with a TI of 3.2 and in this case was a better indicator of teratogenic hazard than some laboratory non-primate tests.

Serotonin when tested in FETAX resulted in a TI of 7.6 and agreed with mice, rat, and human data that serotonin poses a teratogenic hazard. Defects reported in laboratory mice include kidney, abdomen, eye, limb, tail, skull, brain and CNS abnormalities.³⁹⁻⁴⁰ The teratogenic effects of

serotonin in the laboratory rat include anophthalmia, hydrocephalus, exencephaly, omphalocele and vacuolization of myocardial cells.⁴¹ Reddy et al.⁴¹ reported evidence of the effects of serotonin in human pregnancy.

The teratogenicity of 13-cis retinoic acid (Vitamin A) has been observed in all species tested including rat, mouse, rabbit, monkey and also *Xenopus laevis*⁴²⁻⁴³. Nervous system and craniofacial defects are the most common terata reported. Human birth defects as a result of the use of 13-cis retinoic acid are documented and described as a syndrome of central nervous system, aural and cardioaortic defects⁴⁴. J.A.G. Geelen⁴² has published a survey on malformations reported in the literature for Vitamin A and its congeners. FETAX results, a TI of 12.7 for 13-cis retinoic acid, confirm the advice that this treatment for cystic acne should be avoided in pregnant women.

The results of this study indicate that all five compounds agree with the majority of mammalian literature available regarding teratogenicity. Ascorbic acid, cited as a negative teratogen in mammalian literature²⁰⁻²², tested negative in FETAX. Sodium selenate and coumarin, variable positives in mammalian literature^{23-31,32-38}, tested positive in FETAX. Serotonin and 13-cis retinoic acid, listed as positives in mammalian literature^{39-41,42-44}, tested positive in FETAX. Overall, FETAX currently has a predictive accuracy of 89% including compounds tested with metabolic activation.

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TABLE 1.
Developmental Toxicity of Five Compounds Tested with FETAX

| Compound | Test# | LC50 ^a | EC50 ^a | TI | MCIG ^b | MCIG ^c |
|---------------------------------------|-------|--|---|------|---------------------|-------------------|
| Ascorbic acid CAS 50-81-7 | 1 | 19.2 (17.8-20.7) | 11.6 (10.2-13.2) | 1.7 | 10.0 | 52 |
| | 2 | 20.3 (18.7-21.9) | 12.8 (12.4-13.3) | 1.6 | 10.0 | 49 |
| | 3 | 19.6 (18.9-20.3) | 12.0 (10.4-13.8) | 1.6 | 10.0 | 51 |
| Sodium selenate CAS 13410-10-0 | 1 | 0.017 (0.016-0.017) | 0.006 (0.002-0.013) | 3.0 | 0.014 | 82 |
| | 2 | 0.019 (0.017-0.019) | 0.007 (0.006-0.008) | 2.8 | 0.006 | 32 |
| | 3 | 0.027 (0.026-0.029) | 0.009 (0.006-0.012) | 3.1 | 0.008 | 30 |
| Coumarin CAS 91-64-5 | 1 | 0.15 (0.14-0.15) | 0.038 (0.025-0.059) | 4.0 | 0.01 | 7 |
| | 2 | 0.14 (0.13-0.14) | 0.038 NA | 3.5 | 0.05 | 36 |
| | 3 | 0.10 NA | 0.045 (0.037-0.055) | 2.2 | 0.04 | 40 |
| Serotonin CAS 153-98-0 | 1 | 2.74 (2.55-2.93) | 0.35 (0.19-0.66) | 7.8 | 0.25 | 9 |
| | 2 | 3.27 (3.18-3.36) | 0.39 (0.21-0.72) | 8.4 | 0.60 | 18 |
| | 3 | 3.21 NA | 0.48 (0.43-0.54) | 6.7 | 1.00 | 3 |
| 13-Cis retinoic acid CAS 4759-48-2 | 1 | 37x10 ⁻⁹ (25-57x10 ⁻⁹) | 3x10 ⁻⁹ (2-3x10 ⁻⁹) | 18.8 | 7x10 ⁻⁹ | 19 |
| | 2 | 18x10 ⁻⁹ (16-22x10 ⁻⁹) | 2x10 ⁻⁹ (1-3x10 ⁻⁹) | 9.2 | 20x10 ⁻⁹ | NA |
| | 3 | 36x10 ⁻⁹ (34-37x10 ⁻⁹) | 4x10 ⁻⁹ (3-4x10 ⁻⁹) | 10.1 | 10x10 ⁻⁹ | 28 |

a mg/ml with (95% confidence interval).

b Minimum Concentration to Inhibit Growth as mg/L.

c Minimum Concentration to Inhibit Growth as a percent of LC50.

NA Not available.

RESPONSE (PROBIT)

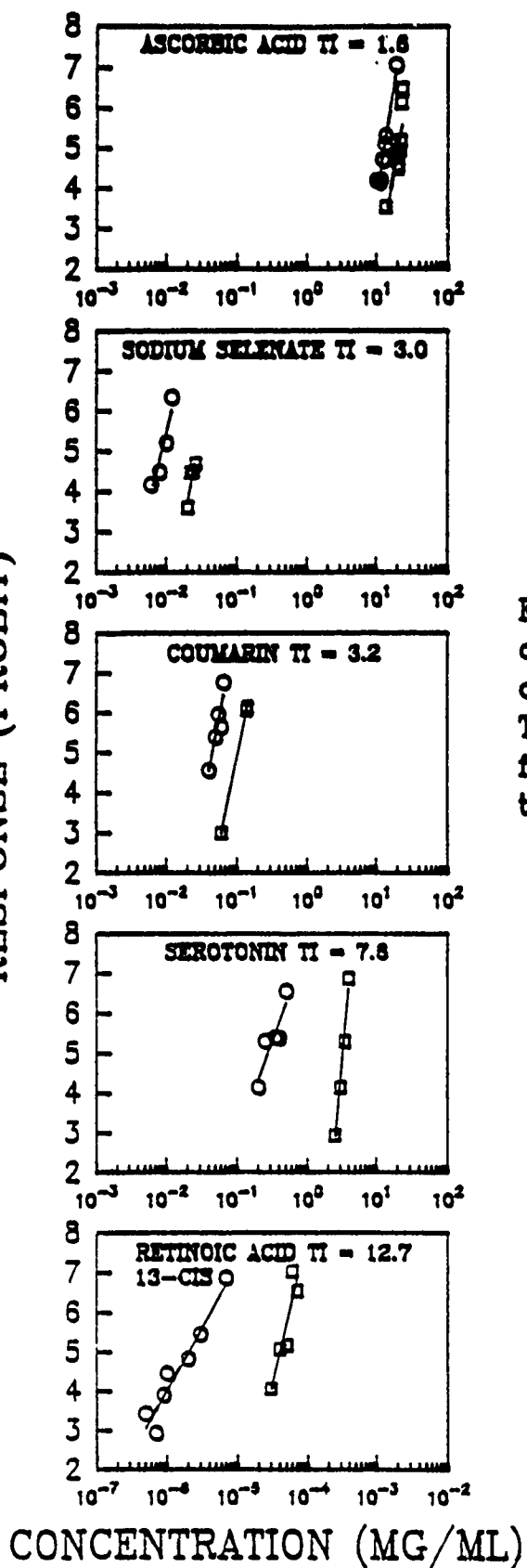


Figure 1. Representative concentration-response curves and respective Teratogenic Index values for the five compounds tested with FETAX

○ Malformation
□ Mortality

PERCENT OF MEAN CONTROL LENGTH

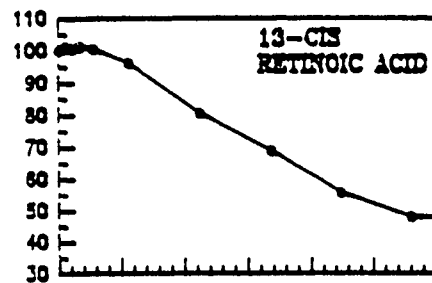
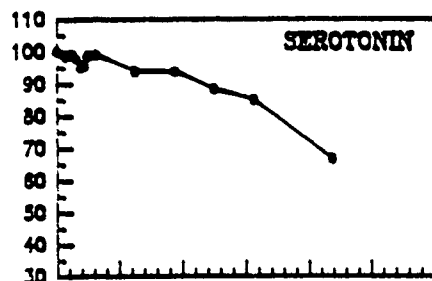
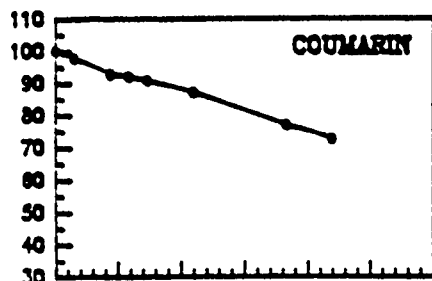
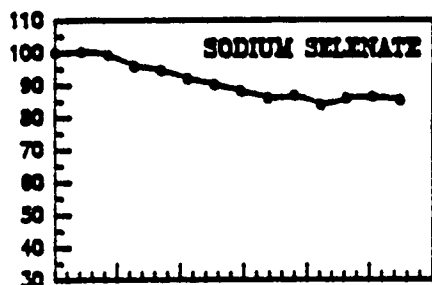
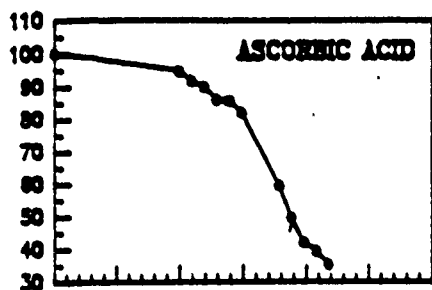


FIGURE 2. Representative embryo growth curves for the five compounds. Concentrations are expressed as percent of the respective compound LC50. Growth is expressed as percent of mean FETAX solution control length.

CONCENTRATION (PERCENT OF LC50)

FETAX Publications from the lab of Dr. John A. Bantle
under contract no. DAMD17-88-C-8031

1. Fort, D.J., James, B.L. and Bantle, J.A., Evaluation of the developmental toxicity of five compounds with the frog embryo teratogenesis assay: *Xenopus* (FETAX). Journal of Applied Toxicology 9(6):377-388, 1989.
2. Bantle, J.A., Fort, D.J., Rayburn, J.R., DeYoung, D.J. and Bush, S.J., Further validation of FETAX: Evaluation of the developmental toxicity of five known mammalian teratogens and non-teratogens. Drug and Chemical Toxicology 13(4):267-282, 1990.
3. Bantle, J.A., Dumont, J.N., Finch, R. and Linder, G., Atlas of abnormalities: a guide for the performance of FETAX. Oklahoma State Publications Department, Publication date: Jan., 1991.

In Press:

4. Rayburn, J.R., Fort, D.J., McNew, R. and Bantle, J.A., Synergism and antagonism induced by three carrier solvents with t-retinoic acid and 6-aminonicotinamide. Bulletin of Environmental Contamination and Toxicology, April, 1991.
5. Fort, D.J., Rayburn, J.R., DeYoung, D.J. and Bantle, J.A., Developmental toxicity testing with frog embryo teratogenesis assay-*Xenopus* (FETAX): Efficacy of Aroclor 1254-induced exogenous metabolic activation system. Drug and Chemical Toxicology, August, 1990.
6. Rayburn, J.R., Fort, D.J., DeYoung, D.J., McNew, R. and Bantle, J.A., Altered developmental toxicity caused by three carrier solvents. Journal of Applied Toxicology.
7. DeYoung, D.J., Bantle, J.A. and Fort, D.J., Assessment of the developmental toxicity of ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid using FETAX. Drug and Chemical Toxicology.

In Preparation

8. Bantle, J.A. and Sabourin, T.D., New standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (FETAX). American Society for Testing and Materials. Fifth draft now out for E.47.01 Aquatic Toxicology ballot. June, 1991.
9. DeYoung, D.J. and Bantle, J.A., Differing sensitivities to sodium acetate, caffeine and 5-flourouracil shown by *Xenopus* and *Pimephales* embryos. Aquatic Toxicology, April, 1991.

Presentations

1. Fort, D.J., Dawson, D.A. and Bantle, J.A., 1988. Preliminary validation of a metabolic activation system for the frog embryo teratogenesis assay-*Xenopus* (FETAX). 9th annual SETAC meeting, Pensacola, FL.
2. Rayburn, J.R., Fort, D.J., James, B.L. and Bantle, J.A., 1988. The use of carrier solvents as an acceptable method of testing water insoluble compounds with FETAX. 77th meeting of the Oklahoma Academy of Science, Chickasha, OK.

3. Fort, D.J., James, B.L. and Bantle, J.A., 1988. Preliminary validation of a metabolic activation system for the frog embryo teratogenesis assay-*Xenopus* (FETAX). 77th meeting of the Oklahoma Academy of Science, Chickasha, OK.
4. Bantle, J.A., 1989. Evaluation of a model system for the detection of developmental toxicants. Invited seminar. Graduate Toxicology Seminar. University of Kentucky, Lexington, KY.
5. Fort, D.J., James, B.L. and Bantle, J.A., 1989. Evaluation of the developmental toxicity of five compounds with the frog embryo teratogenesis assay: *Xenopus* (FETAX) and a metabolic activation system. Ozark-Prairie regional SETAC meeting, Columbia, MO.
6. Rayburn, J.R., Fort, D.J., James, B.L. and Bantle, J.A., 1989. Effects of carrier solvents in FETAX and a proposed method of studying solvent interaction. Ozark-Prairie regional SETAC meeting, Columbia, MO.
7. DeYoung, D.J., Fort, D.J., Rayburn, J.R., Bush, S.J., James, B.L., Work, P.K. and Bantle, J.A., 1989. Validation of FETAX with known mammalian teratogens and nonteratogens. Ozark-Prairie regional SETAC meeting, Columbia, MO.
8. Bantle, J.A., 1989. Validation of FETAX. Advances in *In Vitro* Teratology Conference and Workshop. NIEHS, Research Triangle Park, NC.
9. Fort, D.J., James, B.L. and Bantle, J.A., 1989. Developmental toxicity testing with FETAX and a exogenous metabolic activation system. 10th annual Society of Environmental Toxicology and Chemistry Meetings, Toronto, Canada.
10. DeYoung, D.J., Fort, D.J., Rayburn, J.R., Work, P.K. and Bantle, J.A., 1989. Validation of FETAX with known mammalian teratogens and nonteratogens. 10th annual Society of Environmental Toxicology and Chemistry Meetings, Toronto, Canada.
11. Rayburn, J.R., McNew, R.W. and Bantle, J.A., 1989. The synergistic and antagonistic effects of three carrier solvents on the developmental toxicity of retinoic acid and 6-aminonicotinamide with FETAX. 78th meeting of the Oklahoma Academy of Science, Edmond, OK.
12. Fort, D.J. and Bantle, J.A., 1989. Evaluation of the developmental toxicity of diphenylhydantoin with FETAX: Proteratogen or ultimate teratogen. 78th meeting of the Oklahoma Academy of Science, Edmond, OK.
13. DeYoung, D.J., Fort, D.J., Rayburn, J.R., Work, P.K. and Bantle, J.A., 1989. Validation of FETAX with known mammalian teratogens and nonteratogens. 78th meeting of the Oklahoma Academy of Science, Edmond, OK.
14. Bantle, J.A., 1990. Testing some old drugs using a new developmental toxicity assay. Invited lecture at the University of Connecticut Health Center.
15. Fort, D.J. and Bantle, J.A., 1990. Developmental toxicity testing with FETAX: An overview. SETAC Ozark Prairie-South Central regional meeting, Stillwater, OK.
16. Rayburn, J.R., Fort, D.J. and Bantle, J.A., 1990. The synergism and antagonism effects of the developmental toxicity of t-retinoic acid and 6-aminonicotinamide using FETAX with three carrier solvents. SETAC Ozark Prairie-South Central regional meeting,

- Stillwater, OK.
17. DeYoung, D.J., Fort, D.J., Rayburn, J.R., Hull, M.A. and Bantle, J.A., 1990. Predictive accuracy of the frog embryo teratogenesis assay: *Xenopus* (FETAX). SETAC Ozark Prairie-South Central regional meeting, Stillwater, OK.
18. Bantle, J.A., 1990. FETAX- A developmental toxicity screen. Third Annual Carcinogenicity Review Workshop, Frederick, MD.
19. Bantle, J.A., 1990. Further developments in the FETAX assay. Invited lecture at the University of Connecticut Health Center.
20. Rayburn, J.R., Fort, D.J. and Bantle, J.A., 1990. Effects of solvent-teratogen interaction on developmental toxicity using FETAX. 11th annual Society of Environmental Toxicology and Chemistry Meetings, Arlington, VA.
21. DeYoung, D.J., Fort, D.J., Rayburn, J.R., Hull, M.A. and Bantle, J.A., 1990. Predictive accuracy of the frog embryo teratogenesis assay: *Xenopus* (FETAX). 11th annual Society of Environmental Toxicology and Chemistry Meetings, Arlington, VA.
22. Fort, D.J., Rayburn, J.R. and Bantle, J.A., 1990. Developmental toxicity testing with FETAX: Efficacy of aroclor 1254-induced exogenous metabolic activation systems. 11th annual Society of Environmental Toxicology and Chemistry Meetings, Arlington, VA.
23. Rayburn, J.R., Fort, D.J., DeYoung, D.J. and Bantle, J.A., to be presented April 14-16, 1991. An evaluation of solvent-teratogen interactions using FETAX. ASTM Symposium on Environmental Toxicology and Risk Assessment: Aquatic, Plant, and Terrestrial, Atlantic City, NJ.

Personel Having Received Pay from Contract no. DAMD17-88-C-8031

Name

Dr. John A. Bantle

James R. Rayburn

Donna J. DeYoung

P.K. Work

Mendi A. Hull

Bruce A. Vandelune

Lynne M. Homer

Karmen A. Almgren

Shirley G. Bush

Graduate Degrees Resulting from Contract no. DAMD17-88-C-8031

Three degrees developed from this contract:

- 1) Douglas J. Fort received a Doctor of Philosophy
Dissertation title: Developmental Toxicity Testing with the frog
embryo teratogenesis assay - *Xenopus* (FETAX) and an exogenous
metabolic activation system: Evaluation and Applications.
- 2) James R. Rayburn received a Master of Science
Thesis title: The synergism and antagonism of the developmental
toxicity of t-retinoic acid and 6-aminonicotinamide using FETAX
with 3 carrier solvents.
- 3) Donna J. DeYoung is in the process of completing a Master of Science
Thesis title: FETAX validation and species differences in
developmental toxicity observed with *Xenopus laevis* and *Pimephales
promelas* embryos.

APPENDIX III
RAW DATA

FETAX SUMMARY SHEET (96hr)

COMPOUND AMAZANTH 1 CAS # 915-67-3TEST # DBP 1 TEST UNITS mb/mlFETAX CONTROL MORT. (N,%) 40, 0 FETAX CONT. MALF. (N,%) 40, 0SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —CONTROL LENGTH 0.73 cm MCIG > 4.0LC50 (MORT.) 2.67 95% CONFIDENCE LIMITS 2.13-3.25STATISTICAL TEST USED L/WEC50 (MALF.) 3.53 95% CONFIDENCE LIMITS 3.03-5.17STATISTICAL TEST USED L/WTI —MORTALITYMALFORMATIONSTATISTICAL TEST USEDNOEL 2.5NOEL 2.5DUNNETT'SLOEL 3LOEL 3—METABOLIC ACTIVATIONFETAX CONT. MORT. (N,%) — FETAX CONT. MALF. (N,%) —SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —MAS CONT. MORT. (N,%) — MAS CONT. MALF. (N,%) —MAS+SOL. CONT. MORT. (N,%) — MAS+SOL. CONT. MALF. (N,%) —CYCLOPHOS. CONT. MORT. — CYCLOPHOS. CONT. MALF. —
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. — COMAS+TOX. CONT. MALF. —
() ()MAS+SOLVENT CONTROL LENGTH — cm MCIG —LC50 (MORT.) — 95% CONFIDENCE LIMITS —STATISTICAL TEST USED —EC50 (MALF.) — 95% CONFIDENCE LIMITS —STATISTICAL TEST USED —TI —MORTALITYMALFORMATIONSTATISTICAL TEST USEDNOEL —NOEL ——LOEL —LOEL ——

RAW DATA SUMMARY SHEET (96hr)

COMPOUND AMAZANTH CAS # _____

TEST # 7

CONCENTRATION (mg/ml)

MORTALITY %

MALFORMATION %

FGTAX CONTROL

0

1.5

5

5.3

2

30

7.1

2.5

20

6.3

3

67.5

48.4

3.5

75

70 .

4

97.5

100

FETAX SUMMARY SHEET (96hr)

COMPOUND AMARANTH 2 CAS # 915-67-3

TEST # DBF 2 TEST UNITS MG/ML

FETAX CONTROL MORT. (N,%) 30, 0 FETAX CONT. MALF. (N,%) 80, 5

SOLVENT CONT. MORT. (N,%) - SOLVENT CONT. MALF. (N,%) -

CONTROL LENGTH 0.89 cm MCIG 3.0

LC50 (MORT.) 3.68 95% CONFIDENCE LIMITS 3.47-3.97

EC50 (MALF.) 3.1 STATISTICAL TEST USED L/W

95% CONFIDENCE LIMITS 2.92-3.21

STATISTICAL TEST USED L/W

TI 1.2

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|------------------|-----------------------|
| NOEL <u>2.5</u> | NOEL <u>2.5</u> | <u>DUNNETT'S</u> |
| LOEL <u>2.5</u> | LOEL <u>2.75</u> | <u>1.</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ (4.0 MG/ML) CYCLOPHOS. CONT. MALF. _____ (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ () COMAS+TOX. CONT. MALF. _____ ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

EC50 (MALF.) _____ STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND AMAZANTH CAS #

TEST # 2

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Amaranth CAS # 915-67-3

TEST # 3 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 100, 12.5% FETAX CONT. MALF. (N,%) 88, 12.5%

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

CONTROL LENGTH .84970 cm MCIG 3.75 mg/ml

LC50 (MORT.) 3.8089 95% CONFIDENCE LIMITS (3.60833 - 4.02008)

EC50 (MALF.) 3.91071 STATISTICAL TEST USED Litchfield/Wilcoxon

95% CONFIDENCE LIMITS (3.82413 - 3.99724)

STATISTICAL TEST USED Litchfield/Wilcoxon

TI 0.97

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------------|------------------|-----------------------|
| NOEL <u>3.75</u> | NOEL <u>3.75</u> | <u>Dunnett's</u> |
| LOEL <u>4.00</u> | LOEL <u>4.00</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ (4.0 MG/ML) CYCLOPHOS. CONT. MALF. _____ (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ () COMAS+TOX. CONT. MALF. _____ ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Amaranth

CAS # 915-67-3

TEST # 3

CONCENTRATION (mg/ml)

MORTALITY %

MALFORMATION %

Control

12.75

12.5

2.00

6.00

14.8

2.25

10.00

17.7

2.50

11.00

30.9

2.75

28.00

30.5

3.00

20.00

27.5

3.25

20.00

27.5

3.50

20.00

17.5

3.75

40.00

30.0

4.00

66.00

47.0

4.25

72.00

85.7

4.50

62.00

40.4

4.75

72.00

64.3

5.00

96.00

100.0

5.25

86.00

100.0

FETAX SUMMARY SHEET (96hr)

COMPOUND Aspartame. CAS # 22839-47-0
TEST # 1 TEST UNITS mg/ml
FETAX CONTROL MORT. (N,%) 100, 27 FETAX CONT. MALF. (N,%) 98, 8.15%
SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A
CONTROL LENGTH .83896 cm MCIG 3.0 mg/ml
LC50 (MORT.) Did not reach 95% CONFIDENCE LIMITS _____
EC50 (MALF.) Did not reach STATISTICAL TEST USED _____
95% CONFIDENCE LIMITS _____
STATISTICAL TEST USED _____
TI N/A

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|----------------------|----------------------|------------------------------|
| NOEL <u>> 8.0</u> | NOEL <u>> 8.0</u> | <u>Dunnett's</u> |
| LOEL <u>> 8.0</u> | LOEL <u>> 8.0</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)
COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()
MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
STATISTICAL TEST USED _____
EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
STATISTICAL TEST USED _____
TI _____

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

COMPOUND Aspartame

CAS # 22839-47-0

TEST #

7

Conc. of 50% variation is present in 1 side w/ 50% variation
and 1 side with ^{very} practically no variation.

FETAX SUMMARY SHEET (96hr)

COMPOUND Aspartame CAS # 22839-47-0TEST # 2 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 4% FETAX CONT. MALF. (N,%) 96, 5.2SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH .80861 cm MCIG 7.0 mg/ml (lowest concentration tested)LC50 (MORT.) DID NOT REACH 95% CONFIDENCE LIMITS _____EC50 (MALF.) DID NOT REACH STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI N/A

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL > 10.0NOEL > 10.0DunnettsLOEL > 10.0LOEL > 10.0Dunnetts

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____

(4.0 MG/ML)

(4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____

(

)

(

)

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Aspartame CAS # 22839-47-0

TEST # 2

[illegible]

Dec - next contamination was widespread

FETAX SUMMARY SHEET (96hr)

Det #3

COMPOUND ASPARTAME CAS # 22839-47-0

TEST # def 3 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 200, 19 FETAX CONT. MALF. (N,%) 200, 9

SOLVENT CONT. MORT. (N,%) NA SOLVENT CONT. MALF. (N,%) NA

CONTROL LENGTH 0.87057 cm MCIG 7.0

LC50 (MORT.) 13.91534 95% CONFIDENCE LIMITS 11.92813 - 16.23362

STATISTICAL TEST USED LOW

EC50 (MALF.) 13.14061 95% CONFIDENCE LIMITS 10.95085 - 15.76824

STATISTICAL TEST USED LOW

TI 1.05896

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL -

NOEL -

D

LOEL -

LOEL -

D

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ (4.0 MG/ML) CYCLOPHOS. CONT. MALF. _____ (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ () COMAS+TOX. CONT. MALF. _____ ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Aspartame

CAS #

22839-47-0

TEST #

def 3

[illegible]

Det # 1

FETAX SUMMARY SHEET (96hr)

COMPOUND 5 AC Acetaminophen CAS # 320-67-2TEST # 21 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 17 FETAX CONT. MALF. (N,%) 93, 19.3

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH .94421 cm MCIG .04LC50 (MORT.) .57 95% CONFIDENCE LIMITS .553 - .6379STATISTICAL TEST USED L-W ProbitEC50 (MALF.) .014 95% CONFIDENCE LIMITS .0114 - .017228STATISTICAL TEST USED L-W ProbitTI 42.46

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|----------------|------------------|-----------------------|
| NOEL <u>.1</u> | NOEL <u>A.A.</u> | <u>Dumett's</u> |
| LOEL <u>.4</u> | LOEL <u>.01</u> | <u>Dumett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND 5 AZ Acetyl line

CAS # 320-67-2

TEST # 01

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND 5-Azacytidine CAS # 320-67-2TEST # 2 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 0 FETAX CONT. MALF. (N,%) 100, 7%SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH .90855 cm MCIG (.03 < x < .4 mg/ml)LC50 (MORT.) .6219228 95% CONFIDENCE LIMITS (.5874771 - .6583881)EC50 (MALF.) > .05 95% CONFIDENCE LIMITS Did not reach EC50TI N/A STATISTICAL TEST USED Did not run

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL .5NOEL .035Dunnett'sLOEL .6LOEL .045Dunnett's

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND 5-Azacytidine

CAS # 320-67-2

TEST # 2

| <u>CONCENTRATION (mg/ml)</u> | <u>MORTALITY %</u> | <u>MALFORMATION %</u> |
|------------------------------|--------------------|-----------------------|
| <u>Control</u> | <u>0</u> | <u>7.00</u> |
| <u>.0050</u> | <u>0</u> | <u>6.00</u> |
| <u>.0075</u> | <u>4</u> | <u>19.00</u> |
| <u>.0100</u> | <u>0</u> | <u>10.00</u> |
| <u>.0150</u> | <u>0</u> | <u>6.00</u> |
| <u>.0200</u> | <u>2</u> | <u>16.35</u> |
| <u>.0250</u> | <u>0</u> | <u>16.00</u> |
| <u>.0300</u> | <u>2</u> | <u>20.40</u> |
| <u>.0350</u> | <u>4</u> | <u>23.00</u> |
| <u>.0400</u> | <u>0</u> | <u>26.00</u> |
| <u>.0450</u> | <u>4</u> | <u>33.40</u> |
| <u>.0500</u> | <u>8</u> | <u>21.70</u> |
| <u>.4000</u> | <u>4</u> | <u>100.00</u> |
| <u>.5000</u> | <u>16</u> | <u>100.00</u> |
| <u>.6000</u> | <u>38</u> | <u>100.00</u> |
| <u>.7000</u> | <u>74</u> | <u>100.00</u> |
| <u>.8000</u> | <u>74</u> | <u>100.00</u> |
| <u>.9000</u> | <u>60</u> | <u>100.00</u> |
| <u>1.0000</u> | <u>34</u> | <u>100.00</u> |
| <u>1.2000</u> | <u>30</u> | <u>100.00</u> |
| | | |
| | | |
| | | |

Blind Testing
FETAX SUMMARY SHEET (96hr)

Net "C"

COMPOUND 5-Azacytidine CAS # 320-67-2

TEST # 3 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 100, 16 FETAX CONT. MALF. (N,%) 84, 22.7%

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

CONTROL LENGTH .78234 cm MCIG .1 mg/ml

LC50 (MORT.) .6021179 95% CONFIDENCE LIMITS (.5634072 - .6434883)

STATISTICAL TEST USED Litchfield / u. loxon

EC50 (MALF.) .0669347 95% CONFIDENCE LIMITS (.0621346 - .0715810)

STATISTICAL TEST USED Litchfield / u. loxon

TI 9.0

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL .5

NOEL <.06

Dunnnett's

LOEL .6

LOEL .06

Dunnnett's

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

Blind Testing
RAW DATA SUMMARY SHEET (96hr)

COMPOUND 5-Azarytidine

CAS #

320-67-2

TEST #

3

[illegible]

N - 1

Blind Testing
FETAX SUMMARY SHEET (96hr)

COMPOUND Methotrexate CAS # 59-05-2
TEST # Range TEST UNITS mg/ml
FETAX CONTROL MORT. (N,%) 60, 0% FETAX CONT. MALF. (N,%) 60, 5.0%
SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A
CONTROL LENGTH .91783 cm MCIG .03 mg/ml.

LC50 (MORT.) .7879 95% CONFIDENCE LIMITS (.6567 - .9226)
STATISTICAL TEST USED EPA Probit
EC50 (MALF.) .0185 95% CONFIDENCE LIMITS (.0121 - .0263)
STATISTICAL TEST USED EPA Probit
TI 42.59

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|---------------------|---------------------|------------------------------|
| NOEL <u>DID NOT</u> | NOEL <u>RUN</u> | <u>Not enough variance</u> |
| LOEL <u>DID NOT</u> | LOEL <u>RUN</u> | <u>between groups to run</u> |
| | | <u>Dunnnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)
COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
STATISTICAL TEST USED _____
EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
STATISTICAL TEST USED _____
TI _____

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

Blind Testing

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Methytrixate CAS # 59-05-2

TEST # Range

[illegible]

DET 1

Blind Testing
FETAX SUMMARY SHEET (96hr)

COMPOUND Methoxyacet CAS # 59-05-2

TEST # 1 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 100, 7% FETAX CONT. MALF. (N,%) 93, 8.6%

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

CONTROL LENGTH .82997 cm MCIG .02 mg/ml

LC50 (MORT.) .5078 95% CONFIDENCE LIMITS (.4753 - .5425)

STATISTICAL TEST USED L/W

EC50 (MALF.) .0224 95% CONFIDENCE LIMITS (.0207 - .0241)

STATISTICAL TEST USED EPA PROBIT

TI 22.67

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--------------------|---------------------|-----------------------|
| NOEL <u><.5</u> | NOEL <u><.01</u> | <u>Dunnnett</u> |
| LOEL <u>.5</u> | LOEL <u>.01</u> | <u>Dunnnett</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND: Methotrexate CAS # 59-05-2

TEST # 1

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND c-pseudoephedrine - HCl CAS # 345-78-8TEST # 2 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 2% FETAX CONT. MALF. (N,%) 48, 7.2%SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH .85452 cm MCIG < .20 mg/mlLC50 (MORT.) .4350831 95% CONFIDENCE LIMITS (.3944017 - .4799607)STATISTICAL TEST USED Litchfield / WilcoxonEC50 (MALF.) .2637381 95% CONFIDENCE LIMITS (.2136499 - .3255691)STATISTICAL TEST USED Litchfield / WilcoxonTI 1.65

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------|-----------------------|
| NOEL <u>.35</u> | NOEL <u>.20</u> | <u>Dunnett's</u> |
| LOEL <u>.43</u> | LOEL <u>.25</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND c-pseudoephedrine-HCl CAS # 343-78-8

TEST # 2

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND d-pseudoephedrine HCl CAS # 345-78-8TEST # 3 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 9% FETAX CONT. MALF. (N,%) 91, 8.8%SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH .86450 cm MCIG < .20 mg/mlLC50 (MORT.) .4170759 95% CONFIDENCE LIMITS (.4066924 - .4277246)EC50 (MALF.) ~.225 STATISTICAL TEST USED Litchfield/Wilcoxon

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI ~ 1.85

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL .35NOEL < .20Dunnett'sLOEL .40LOEL < .20Dunnett's

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____

(4.0 MG/ML)

(4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____

(

)

(

)

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

Blind Testing

RAW DATA SUMMARY SHEET (96hr)

COMPOUND d-pseudoephedrine HCl CAS # 345-78-8

TEST # 3

[illegible]

Blind Testing
FETAX SUMMARY SHEET (96hr)

Det # 3

COMPOUND d-pseudoephedrine - HCl CAS # 345-78-8

TEST # 4 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 100, 5% FETAX CONT. MALF. (N,%) 95, 8.3%

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

CONTROL LENGTH .83621 cm MCIG < .150 mg/ml

LC50 (MORT.) .394036 95% CONFIDENCE LIMITS (.3873004 - .4008804)
 EC50 (MALF.) .2105226 95% CONFIDENCE LIMITS (.2049941 - .2162002)
 TI 1.87 STATISTICAL TEST USED Litchfield/Wilcoxon

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL <u>.300</u> | NOEL <u>.150</u> | <u>Dunnett's</u> |
| LOEL <u>.375</u> | LOEL <u>.175</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 EC50 (MALF.) _____ STATISTICAL TEST USED _____
 TI _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

CAS # 345-78-8

TEST # 4

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND d- pseudophedrine - HCl / MBAS # 345-78-8TEST # MAS 1 TEST UNITS ug/mlFETAX CONTROL MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 0SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH .94360 cm MCIG 205LC50 (MORT.) > 425 95% CONFIDENCE LIMITS _____EC50 (MALF.) < 205 STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI > 2.0

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL DID NOT RUN NOEL _____LOEL DID NOT RUN LOEL _____

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 0SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/AMAS CONT. MORT. (N,%) 40, 0 MAS CONT. MALF. (N,%) 40, 2.5MAS+SOL. CONT. MORT. (N,%) N/A MAS+SOL. CONT. MALF. (N,%) N/ACYCLOPHOS. CONT. MORT. N/A CYCLOPHOS. CONT. MALF. N/A
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. N/A COMAS+TOX. CONT. MALF. N/A
() ()MAS+~~SOLVENT~~ CONTROL LENGTH .90321 cm MCIG _____LC50 (MORT.) > 425 95% CONFIDENCE LIMITS _____EC50 (MALF.) > 425 STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI N/A

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL DID NOT RUN NOEL _____LOEL DID NOT RUN LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Pseudoephedrine-HCl

CAS # 345-78-8

TEST # MAS 1

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND d-pseudoephedrine-HCl CAS # 345-78-8

TEST # MAS # 2 TEST UNITS ug/ml

FETAX CONTROL MORT. (N,%) 30, 0 FETAX CONT. MALF. (N,%) 30, 0

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

CONTROL LENGTH .95018 cm MCIG 225

LC50 (MORT.) > 425 95% CONFIDENCE LIMITS _____

EC50 (MALF.) < 225 STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI > 1.89

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL DID NOT RUN NOEL _____

LOEL DID NOT RUN LOEL _____

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 30, 0 FETAX CONT. MALF. (N,%) 30, 0

SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/A

MAS CONT. MORT. (N,%) 30, 3.34 MAS CONT. MALF. (N,%) 29, 10.5

MAS+SOL. CONT. MORT. (N,%) N/A MAS+SOL. CONT. MALF. (N,%) N/A

CYCLOPHOS. CONT. MORT. N/A CYCLOPHOS. CONT. MALF. N/A

(4.0 MG/ML)

(4.0 MG/ML)

COMAS+TOX. CONT. MORT. 30, 0 COMAS+TOX. CONT. MALF. 30, 10.0

(425 ug/ml)

()

MAS+SOLVENT CONTROL LENGTH .86480 cm MCIG 225

LC50 (MORT.) > 425 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) > 425 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI N/A

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL DID NOT RUN NOEL _____

LOEL DID NOT RUN LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Pseudoephedrine-HCl CAS # 345-78-8

TEST # MAS 2

[illegible]

RAW DATA SUMMARY SHEET (96hr) by Brenda James

COMPOUND Ascorbic Acid

CAS #

TEST #

Def #1

VENDOR/LOT #

| CONCENTRATION (mg/ml) | MORTALITY % | MALFORMATION % |
|-----------------------|-------------------------|--------------------------|
| control | 1, 4 } .05, .20 } .11 | 2, 1 } .10, .05 } .075 |
| control | 2, 2 } .10, .10 } | 1, 1 } .05, .05 } |
| 10.0 | 1, 0 } .05, .0 = .025 | 3, 5 } .15, .25 = .20 |
| 11.0 | 5, 0 } 0, .0 = 0 | 9, 5 } .45, .25 = .35 |
| 12.0 | 0, 0 } 0, .0 = 0 | 4, 6 } .20, .30 = .25 |
| 13.0 | 1, 0 } .05, .0 = .025 | 18, 17 } .90, .85 = .88 |
| 14.0 | 0, 0 } 0, .0 = 0 | 20, 20 } .40, .40 = 1.0 |
| 15.0 | 0, 2 } 0, .10 = .05 | 20, 18 } .40, .38 = .39 |
| 18.0 | 3, 2 } .15, .10 = .13 | 17, 18 } .35, .35 = .35 |
| 19.0 | 1, 5 } .05, .25 = .15 | 19, 15 } .35, .30 = .325 |
| 20.0 | 6, 9 } .30, .45 = .375 | 14, 11 } .28, .22 = .25 |
| 21.0 | 12, 17 } .60, .85 = .73 | 8, 3 } .20, .11 = .155 |
| 22.0 | 20, 17 } 1.0, .85 = .93 | - 3 3 } .0, .1 = .05 |
| 23.0 | 26, 20 } 1.0, 1.0 = 1.0 | |

20 per dish

RAW DATA SUMMARY SHEET (96hr) by Brenda James

COMPOUND Ascorbic Acid CAS # _____

TEST # Def #2 VENDOR/LOT # _____

| CONCENTRATION (mg/ml) | MORTALITY % | | MALFORMATION % | |
|-----------------------|-------------|-------------------|----------------|--------------------|
| <u>control</u> | 1,4 | 29 .05, .20 | 0,1 | 7 0, .06 } .03 |
| <u>control</u> | 3,1 | 15, .07 } .11 | 1,6 | 5 2 .06, 0 } .03 |
| <u>10</u> | 1,0 | 0 0, 0 = 0 | 4,2 | 6 .27, .13 = .25 |
| <u>11</u> | 1,2 | 3 .07, .13 = .10 | 1,4 | 5 .07, .03 = .19 |
| <u>11.5</u> | 0,1 | 1 0, .07 = .03 | 3,3 | 6 .20, .21 = .21 |
| <u>12</u> | 0,1 | 1 0, .07 = .03 | 4,7 | 11 .27, .50 = .35 |
| <u>12.5</u> | 0,0 | 0 0, 0 = 0 | 8,3 | 11 .53, .20 = .37 |
| <u>13</u> | 0,0 | 0 0, 0 = 0 | 8,8 | 16 .53, .53 = .53 |
| <u>13.5</u> | 2,0 | 2 .13, 0 = .07 | 9,8 | 17 .69, .53 = .61 |
| <u>19</u> | 5,8 | 13 .33, .53 = .43 | 10,7 | 17 1.00 1.00 = 1.0 |
| <u>19.5</u> | 2,8 | 10 .13, .53 = .33 | 13,7 | 20 |
| <u>20</u> | 5,4 | 9 .33, .27 = .30 | 10,11 | 21 |
| <u>20.5</u> | 5,5 | 10 .33, .33 = .33 | 10,10 | 20 |
| <u>21</u> | 9,5 | 14 .60, .33 = .47 | 6,10 | 16 |
| <u>21.5</u> | 8,6 | 14 .53, .40 = .47 | 7,9 | 16 |
| <u>22</u> | 5,9 | 17 .53, .60 = .57 | 7,6 | 13 |
| <u>22.5</u> | 14,12 | 26 .93, .80 = .87 | 1,3 | 4 |
| <u>23</u> | 15,15 | 30 1.0, 1.0 = 1.0 | — | — |
| | | | | |
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| | | | | |

20 rods/dish
control

15/dish
with ...

RAW DATA SUMMARY SHEET (96hr)

COMPOUND

Sodium Selenate

CAS #

dry work

TEST #

def # 1

VENDOR/LOT #

CONCENTRATION 1 mg/ml

MORTALITY %

MALFORMATION %

control

0,0 0,0

1,3 0,4

control

1,2 3 0,4 0,8 = .03

6,4 14 0,25 0,17 = .14

.002

2,2 = 4 0,8 0,8 = .08

2,11 = 14 0,13 0,48 = .30

.004

1,0 = 1 0,4 0 = .02

13,6 = 19 0,54 0,24 = .39

.006

2,1 = 3 0,5 0,4 = .06

2,3 = 5 0,09 0,13 = .11

.008

1,0 = 1 0,4 0 = .02

10,19 = 29 0,42 0,76 = .59

.01

2,2 = 4 0,2 0,5 = .08

23,9 = 32 1,00 0,39 = .70

.012

2,2 = 4 0,8 0,8 = .08

18,14 = 32 0,78 0,61 = .70

.014

3,2 = 5 0,2 0,8 = .10

22,21 = 43 1,00 0,91 = .96

.016

6,14 = 20 0,24 0,56 = .40

19,11 = 30 1,00 1,00 1,00

.018

13,20 = 33 0,52 0,80 = .66

12,5 = 17

.02

17,23 = 40 0,68 0,92 = .80

8,2 = 10

.022

24,25 = 49 0,96 1,00 = .98

1 = 1

.024

24,25 = 49 0,96 1,00 = .98

1 = 1

.026

25,25 = 50 1,00 1,00 = 1.00

— = —

~.2

~.5

+

~.7

COMPOUND Sodium Selenate CAS # any work.
TEST # def # 2 VENDOR/LOT # _____

MALFORMATION 8

$$0,07 \quad | \quad 0,07 \quad \} 0$$

| | | | |
|-----|-----|-------|-------|
| 1,1 | } 3 | 04,04 | } .03 |
| 1,0 | | 04,0 | |

$$1, 1 = \underline{2} \quad | \quad .04, .04 = .04$$
$$\underline{1,1 = 2} \quad 104,04 : 04$$
$$0, 0 : \underline{0 \mid 0 \ 0} = 0$$
$$\underline{2, 7 = 14} \quad | \quad 28, 28 = .28$$
$$0, 0 : 0 \quad | \quad 0 \quad 0 = 0$$
$$\underline{10, 10 = 20} \quad \underline{40, 40 = 40}$$
$$1, 0 = \underline{1} \mid \underline{.04}, 0 = .02$$
$$\underline{11, 13 = 24, 41, 52 = .49}$$
$$1, 0 = \underline{1} \mid \underline{.04, 6} = .02$$
$$\underline{18, 18 = 36, 75, 72 = 72}$$
$$1, 5 = \underline{6} \mid .04, 20 = .12$$
$$\underline{17, 14 = 31} \quad \underline{17, 70 = 70}$$
$$3, 6 = 9, 12, 24 = 18$$
$$\underline{17,18 = 35,77,95 = .85}$$
$$6, 6: 12 \mid 24, 24 = 24$$
$$\underline{17, 19 = 36, 89, 1.00 = .95}$$
$$13, 12 = 25, 52, 48 = .50$$
$$\underline{9, 12 = 21, 75, 92 = .84}$$
$$\underline{18, 13 = 31} \quad \underline{72, 52 = 124}$$
$$\underline{7, 12 = 19} \quad \underline{100, 100 = 200}$$
$$12, 14, \underline{26}, 48, \underline{56} = 52$$

13, 11 = 24

$$22, 16 \cdot 38, 88, 64 = .76$$
$$\underline{3, 9 = 12}$$
$$20,15 = \underline{35} \mid 80,60 = .70$$
$$\underline{5, 10 = 15}$$

1

!

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Sodium Selenate CAS # by work
TEST # Def # 3 VENDOR/LOT # 13410-01-0

[illegible]

25/11/19

RAW DATA SUMMARY SHEET (96hr) by DeYoung

COMPOUND Coumarin CAS #

TEST # def #1 11/18/88 VENDOR/LOT # _____

| CONCENTRATION (mg/ml) | # | MORTALITY % | % | MALFORMATION % |
|-----------------------|-----------|-------------|------|---------------------------|
| control | 00 = 0 | 0.07 = 0 | | 1072 04, 0 |
| control | 00 0 | ... } = 0 | | 0152 0.04 = 0.02 |
| .01 | 0, 0 = 0 | 0 0 = 0 | | 2, 6 8 18, 24 = 16 |
| .02 | 0, 0 0 | 0 0 0 | | 6, 3 9 24, 12 = 18 |
| .03 | 0, 0 0 | 0 0 0 | | 6, 8 14 24, 32 = 28 |
| .04 | 1, ^ | 1, 04, 0 | .02 | 6, 13 19 25, 52 = .39 |
| .05 | 0, 2 2 | 0, 08 | .04 | 14, 14 28 56, 61 = .58 |
| .075 | 0, 2 2 | 0, 08 | .04 | 25, 23 48 100, 100 = 1.00 |
| .10 | 0, 0 0 | 0, 0 | .00 | 25, 25 50 |
| .125 | 5 2 7 | 2, 08 | .14 | 20, 23 47 |
| .15 | 14, 14 28 | 56, 56 | .56 | 11, 11 22 |
| .17 | 15, 23 37 | 60, 92 | .74 | 10, 2 12 |
| .19 | 25, 23 48 | 100, 92 | .96 | 0, 2 2 |
| .20 | 25, 25 50 | 100, 100 | 1.00 | -- |
| DMSO control | 0 0 0 | 0, 0 | .0 | 1, 1 2 04, 04 = .0 |

CAS

VENDOR/LOT #

MALFORMATION %

$$\frac{1,1}{0,0} \} 2 \quad \left| \quad \frac{.05, .06}{.0, .0} \right\} .06$$

2,1) 10,05)

| | |
|------|----------|
| 0,05 | 1,0,1,05 |
|------|----------|

$$3,2 \quad \underline{5} \quad \underline{.15.10} = .12.5$$
$$\frac{4,1}{5} \cdot 24,053 = 0,14$$
$$1, 4 \quad \underline{5} \quad .05 \cdot 20 = 12, 5$$

| | | | |
|-----|---|--------|----|
| 1.5 | 6 | 05, 31 | 17 |
|-----|---|--------|----|

$$2, 1 \quad 3 \quad | \quad .10 \quad .05 = .075$$
$$3.3 \quad 6 \mid 17, 16 = 16$$
$$0,1 \quad | \quad .05 = .025$$

3, 4 7 | 15, 21 - 18

$$0,0 \quad 0 \quad | \quad 0,0 = 0$$

2,8 15 10 40 25

$$2 \quad 3 \quad 5 \quad | \quad 10 \quad 15 = 12 \quad 5$$

18.17 35 | 1.00.1.06 = 1.02

19.19 38 | .95 .95 = .95

| | | |
|------|---|---|
| 1, 1 | 2 | 1 |
|------|---|---|

$$20, 30, 40, 100, 100 = 100$$

| | | | |
|---|---|--|--|
| — | — | | |
|---|---|--|--|

$$20 \cdot 20 \cdot 40 \cdot 100 \cdot 100 = 1.000.000$$

| | | |
|---|---|--|
| — | — | |
|---|---|--|

20, 20 40 100 1.00 = 1.00

— — | —

$$0 \quad 0 \quad 0 \quad | \quad 0 \quad 0 = 0$$
$$2.1 \cdot 3 \cdot 10^{-5} = .075$$

20/disch

RAW DATA SUMMARY SHEET (96hr) by DeYoung

COMPOUND Coumarin CAS # _____

TEST # Def # 4 3/16/89 VENDOR/LOT # _____

| CONCENTRATION (mg/ml) | MORTALITY % | | MALFORMATION % | |
|-----------------------|-------------|-------------------|----------------|------------------|
| control | 0,0 | 0.0 | 0,0 | 0.0 |
| control | 0,0 | 0.0 | 1,0 | 0.05 |
| .001 | 0,3 | 0.15 = .075 | 5,1 | 0.25, .06 = .16 |
| .04 | 0,0 | 0.0 = 0 | 9,4 | 0.45, .20 = .33 |
| .05 | 0,0 | 0.0 = 0 | 14,12 | 0.70, .60 = .65 |
| .055 | 0,0 | 0.0 = 0 | 15,18 | 0.75, .90 = .85 |
| .06 | 0,1 | 0.1, .05 = .025 | 15,14 | 0.75, .74 = .74 |
| .065 | 0,0 | 0.0 = 0 | 20,20 | 1.00, 1.00 = 1.0 |
| .07 | 0,0 | 0.0 = 0 | 20,20 | 40 |
| .075 | 0,0 | 0.0 = 0 | 20,20 | 40 |
| .08 | 0,0 | 0.0 = 0 | 20,20 | 40 |
| .137 | 20,9 | 1.05, .45 = .725 | 0,11 | 11 |
| .14 | 20,20 | 1.00, 1.00 = 1.00 | --- | --- |
| .143 | ↓ ↓ ↓ | | --- | --- |
| .145 | | | --- | --- |
| .15 | | | --- | --- |
| .153 | | | --- | --- |
| .155 | | | --- | --- |
| DMSO | 1,0 | 0.05, 0 = .025 | 0,0 | 0 |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

20/dish

RAW DATA SUMMARY SHEET (96hr) by Shirley Bush

COMPOUND Serotonin CAS # _____
 TEST # _____ VENDOR/LOT # _____

| CONCENTRATION (mg/ml) | MORTALITY % | | MALFORMATION % | |
|-----------------------|-------------|----------------|----------------|-------------------|
| Control | 0,0 | 0,0 | 6,4 | 24,16 |
| Control | 0,0 | 0,0 | 3,1 | 12,04 |
| .2 | 0,1 | .04 = .02 | 15,14 | 29,63 = .59 |
| .4 | 1,2 | .04, .08 = .06 | 18,21 | 39,75,91 = .82 |
| .6 | 1,2 | .04, .08 = .06 | 24,23 | 47,1.0, 1.0 = 1.0 |
| .8 | 1,0 | .04, 0 = .02 | 24,25 | 49 |
| 1.0 | 1,0 | .04, 0 = .02 | 24,25 | 49 |
| 1.5 | 0,2 | 0, .08 = .04 | 25,23 | 48 |
| 2.0 | 2,2 | .04, .04 = .08 | 23,23 | 46 |
| 2.5 | 0,1 | 0, .04 = .02 | 25,24 | 49 |
| 3.0 | 3,7 | .12, .28 = .20 | 22,18 | 40 |
| 3.5 | 14,17 | .56, .68 = .62 | 1,8 | 19 |
| 4.0 | 25,25 | 1.0, 1.0 = 1.0 | | |
| Control | | Rescore | 3,4 | 8,12,16 |
| .2 | | | 1,0 | 8,04,0 |
| .4 | | | 2,8 | 10, .08, .333 |
| .6 | | | 15,14 | 29,63, .61 |
| .8 | | | 16,15 | 31, .67, .65 |
| 1.0 | | | 16,16 | 32, .67, .64 |
| 1.5 | | | 24,25 | 49, 1.00, 1.00 |
| 2.0 | | | | |
| | | | | |
| | | | | |
| | | | | |

RAW DATA SUMMARY SHEET (96hr) by DeYoung

COMPOUND Serotonin

CAS # _____

TEST # Def #3

VENDOR/LOT # _____

CONCENTRATION (mg/ml)

MORTALITY %

MALFORMATION %

| | | | | |
|---------|-------|----|----------|--------|
| control | 0,0 | 0 | .0, .0 | .0 |
| control | 0,0 | 0 | .0, .0 | .0 |
| .1 | 0,1 | 1 | .0, .04 | = .02 |
| .2 | 1,0 | 1 | .04, .0 | = .02 |
| .25 | 0,0 | 0 | .0, .0 | = .0 |
| .3 | 0,0 | 0 | .0, .0 | = .0 |
| .35 | 0,1 | 1 | .0, .04 | = .02 |
| .4 | 0,0 | 0 | .0, .0 | = .0 |
| .5 | 0,0 | 0 | .0, .0 | = .0 |
| 1 | 1,0 | 1 | .04, .0 | = .02 |
| 1.5 | 0,0 | 0 | .0, .0 | = .0 |
| 2 | 1,0 | 1 | .04, .0 | = .02 |
| 2.5 | 1,1 | 2 | .04, .04 | = .04 |
| 3.5 | 13,21 | 34 | .52, .84 | = .68 |
| 4 | 25,25 | 50 | 1.0, 1.0 | = 1.00 |

| | | | |
|-------|-----|----------|-------|
| 1,1 | 2,4 | .04, .04 | = .04 |
| 1,1 | 3 | .04, .04 | = .04 |
| 1,0 | 1 | .04, .0 | = .02 |
| 2,1 | 3 | .08, .04 | = .06 |
| 1,2 | 3 | .04, .08 | = .06 |
| 1,2 | 3 | .04, .08 | = .06 |
| 3,1 | 4 | .12, .04 | = .08 |
| 3,5 | 8 | .12, .20 | = .16 |
| 8,6 | 14 | .32, .24 | = .29 |
| 9,10 | 19 | .38, .46 | = .37 |
| 13,10 | 23 | .52, .40 | = .46 |
| 17,18 | 35 | .71, .72 | = .71 |
| 21,23 | 44 | .88, .96 | = .92 |
| 12,4 | 16 | 1.0, 1.0 | = 1.0 |

Rescore

| | | | | |
|---------|--|--|--|--|
| Control | | | | |
| .1 | | | | |
| .2 | | | | |
| .25 | | | | |
| .3 | | | | |
| .35 | | | | |
| .4 | | | | |
| .5 | | | | |
| 1 | | | | |

| | | | |
|-------|-----|------------|--------|
| 1,1 | 2,4 | .04, .04 | = .04 |
| 1,1 | 3 | .04, .04 | = .04 |
| 5,1 | 6 | .20, .04 | = .12 |
| 4,4 | 8 | .17, .16 | = .16 |
| 5,8 | 13 | .20, .32 | = .26 |
| 8,4 | 12 | .32, .16 | = .24 |
| 9,8 | 17 | .36, .33 | = .35 |
| 9,11 | 20 | .36, .44 | = .40 |
| 10,11 | 21 | .40, .44 | = .42 |
| 24,25 | 49 | 1.00, 1.00 | = 1.00 |

RAW DATA SUMMARY SHEET (96hr) by DeYoung

COMPOUND 13-Cis Retinoic acid CAS # _____

TEST # D #1 VENDOR/LOT # _____

[illegible]

RAW DATA SUMMARY SHEET (96hr) by DeYoung

COMPOUND 13-Cis Retinoic Acid CAS #

TEST # Def #2 VENDOR/LOT # _____

| CONCENTRATION $\mu\text{g/ml}$ | MORTALITY % | | MALFORMATION % | |
|--------------------------------|-------------|----------|----------------|------|
| control | 1, 3 | 7 | 5, 4 | 16 |
| control | 3, 0 | .04, .12 | .21, .18 | .17 |
| .5 | 3, 0 | .13, .0 | .045, .24 | |
| .9 | 2, 3 | 5 | .08, .12 | .10 |
| 1 | 0, 0 | 0 | .10, .10 | .0 |
| 2 | 1, 0 | 1 | .04, .0 | .02 |
| 3 | 0, 0 | 0 | .0, .0 | .0 |
| 7 | 0, 1 | 1 | .0, .04 | .02 |
| 20 | 0, 3 | 3 | .10, .12 | .06 |
| 30 | 8, 20 | 28 | .32, .80 | .56 |
| 40 | 13, 22 | 35 | .52, .88 | .70 |
| 50 | 25, 25 | 50 | 1.0, 1.0 | 1.00 |
| 60 | | | | |
| 120 | | | | |
| 140 | | | | |

Def # 1

FETAX SUMMARY SHEET (96hr)

COMPOUND Triethylene Glycol CAS # 112-27-6TEST # Def 1 TEST UNITS μg/kgFETAX CONTROL MORT. (N,%) 80, 1.3 FETAX CONT. MALF. (N,%) 79, 1.3SOLVENT CONT. MORT. (N,%) NA SOLVENT CONT. MALF. (N,%) NACONTROL LENGTH 0.85 cm MCIG 1.75LC50 (MORT.) 2.4 95% CONFIDENCE LIMITS 2.02 - 2.85EC50 (MALF.) 2.07 STATISTICAL TEST USED Litchfield-Wilcoxon95% CONFIDENCE LIMITS 2.01 - 2.13STATISTICAL TEST USED Litchfield-WilcoxonTI 1.14

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL 2.5NOEL 1.75Dunnett'sLOEL ~~2.5~~ 2.75LOEL 2.0Dunnett's

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND: Triethylene Glycol A CAS # 112-27-6

TEST # Det 1

[illegible]

FETAX SUMMARY SHEET (96hr)

Def = 2

COMPOUND Triethylene Glycol CAS # 112-27-6TEST # Def 2 TEST UNITS v/v %FETAX CONTROL MORT. (N, %) 80, 3.8% FETAX CONT. MALF. (N, %) 80, 0%SOLVENT CONT. MORT. (N, %) NA SOLVENT CONT. MALF. (N, %) NACONTROL LENGTH 1.909631 cm MCIG 1.8 %LC50 (MORT.) 2.76 % 95% CONFIDENCE LIMITS 2.7 - 2.82EC50 (MALF.) 2.41 % STATISTICAL TEST USED Litchfield - Wilcoxon95% CONFIDENCE LIMITS 2.37 - 2.45STATISTICAL TEST USED Litchfield - WilcoxonTI 1.14

MORTALITY

NOEL 2.4 %LOEL 2.5 %

MALFORMATION

NOEL 2.0 %LOEL 2.1 %

STATISTICAL TEST USED

DunnetsDunnets

METABOLIC ACTIVATION

FETAX CONT. MORT. (N, %) _____ FETAX CONT. MALF. (N, %) _____

SOLVENT CONT. MORT. (N, %) _____ SOLVENT CONT. MALF. (N, %) _____

MAS CONT. MORT. (N, %) _____ MAS CONT. MALF. (N, %) _____

MAS+SOL. CONT. MORT. (N, %) _____ MAS+SOL. CONT. MALF. (N, %) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

NOEL _____

LOEL _____

MALFORMATION

NOEL _____

LOEL _____

STATISTICAL TEST USED

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Triethylene Glycol Df2 CAS # 112-27-6

TEST # Def 2

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Triethylene Glycol CAS # 112-27-6

TEST # 3 TEST UNITS V/V%

FETAX CONTROL MORT. (N,%) 100 FETAX CONT. MALF. (N,%) 93, 12

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH 30943 cm MCIG 1.7

LC50 (MORT.) 2.19 95% CONFIDENCE LIMITS 2.065 - 2.32

EC50 (MALF.) 2.05 STATISTICAL TEST USED Likelihood - wilcoxon

95% CONFIDENCE LIMITS 1.989 - 2.113

STATISTICAL TEST USED Likelihood - wilcoxon

TI 1.069

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------------|-----------------|-----------------------|
| NOEL <u>2.2%</u> | NOEL <u>2.7</u> | <u>Likelihood</u> |
| LOEL <u>2.4</u> | LOEL <u>-</u> | <u>Benard's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. (4.0 MG/ML) _____ CYCLOPHOS. CONT. MALF. (4.0 MG/ML) _____

COMAS+TOX. CONT. MORT. () _____ COMAS+TOX. CONT. MALF. () _____

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Triethylene Glycol CAS # 112-27-6
TEST # 3

TEST # 3

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND ACETON 6 CAS # 67-64-1
 TEST # 1 TEST UNITS mg (v/v)
 FETAX CONTROL MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 1.9
 SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —
 CONTROL LENGTH 0.9 cm MCIG 1.25
 LC50 (MORT.) 2.17 95% CONFIDENCE LIMITS 2.09 - 2.25
 STATISTICAL TEST USED LD
 EC50 (MALF.) 1.36 95% CONFIDENCE LIMITS 1.29 - 1.43
 STATISTICAL TEST USED LD
 TI 1.6

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------------|------------------|-----------------------|
| NOEL <u>1.75</u> | NOEL <u>1.25</u> | <u>Dunn + TT's</u> |
| LOEL <u>2</u> | LOEL <u>1.5</u> | |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
 MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
 CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)
 COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND

ACETONE

CAS #

TEST #

1

CONCENTRATION (%)

MORTALITY %

MALFORMATION %

CONTROL3.752.61016.71.56.742.9213.31002.536.71003100—3.5100—4100—4.5100—5100—

FETAX SUMMARY SHEET (96hr)

COMPOUND ACETONE CAS # 67-64-1
 TEST # 2 TEST UNITS (%) V/V

FETAX CONTROL MORT. (N,%) 80, 3.75 FETAX CONT. MALF. (N,%) 76, 2.6

SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —

CONTROL LENGTH 0.86 cm MCIG 1.5

LC50 (MORT.) 2.49 95% CONFIDENCE LIMITS 2.10 - 2.95

STATISTICAL TEST USED 4W

EC50 (MALF.) 1.41 95% CONFIDENCE LIMITS 1.04 - 1.36

STATISTICAL TEST USED 4W

TI 1.8

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL 2.0

NOEL 1.0

DUNNETT'S

LOEL 2.5

LOEL 1.5

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

COMPOUND

ACFTON 5

CAS #

TEST #

2

CONCENTRATION (%)

MORTALITY %

MALFORMATION %

CONTROL



1.9

1.0



5

1.25-

0

20

1.5-

5

63.9

1.75

5

152

2

20

102

2.25

60

100

2.5

90

100

2.75

150

107

3

100

100

FETAX SUMMARY SHEET (96hr)

COMPOUND 4-Fluorophenol CAS # 67-64-1
 TEST # 3 TEST UNITS V/V %
 FETAX CONTROL MORT. (N,%) 30.0 FETAX CONT. MALF. (N,%) 20, 23.7
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 CONTROL LENGTH .85-84 cm MCIG 1
 LC50 (MORT.) 1.92 95% CONFIDENCE LIMITS 1.9 - 2.14
 EC50 (MALF.) 1.064 STATISTICAL TEST USED L-W
 95% CONFIDENCE LIMITS .961 - 1.17
 STATISTICAL TEST USED L-W
 TI 1.83

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------------|-----------------|-----------------------|
| NOEL <u>N.F.</u> | NOEL <u>1.2</u> | <u>Dunn's</u> |
| LOEL <u>N.F.</u> | LOEL <u>1.3</u> | <u>Dunn's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
 MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
 CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)
 COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()
 MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND

CAS #

TEST #

CONCENTRATION (

)

MORTALITY %

MALFORMATION %

Control

0

23.75

.5

2.5

23.03

.7

5

17.50

.1

0

32.50

1

0

37.5

1.2

2.5

48.52

1.3

0

60

1.5

0

92.5

1.7

7.5

100

1.9

25

100

2

70

100

2.5

100

—

FETAX SUMMARY SHEET (96hr)

COMPOUND DMSO CAS # 67-68-5TEST # 1 TEST UNITS (%) v/vFETAX CONTROL MORT. (N,%) 80, 3.8 FETAX CONT. MALF. (N,%) 77, 1.3SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —CONTROL LENGTH 0.91 cm MCIG 1.4LC50 (MORT.) 1.81 95% CONFIDENCE LIMITS 1.75 - 1.87STATISTICAL TEST USED L/WEC50 (MALF.) 1.40 95% CONFIDENCE LIMITS 1.32 - 1.48STATISTICAL TEST USED L/WTI 1.3

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL 1.4NOEL 1.2DUNN-SIDAK'SLOEL 1.6LOEL 1.4

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND

DMSO

CAS #

TEST #

1

CONCENTRATION (%)

MORTALITY %

MALFORMATION %

CONTROL3.751.310101.27.516.21.40401.617.581.81.840100277.51002.2100—2.4100—2.6100—2.8100—3100—

FETAX SUMMARY SHEET (96hr)

COMPOUND DM-50 CAS # 67-68-5
 TEST # 2 TEST UNITS V/V %
 FETAX CONTROL MORT. (N,%) 80, 6.67% FETAX CONT. MALF. (N,%) 56, 7.16
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 CONTROL LENGTH .848 cm MCIG 1.7

LC50 (MORT.) 1.77 95% CONFIDENCE LIMITS 1.25 - 1.329
 STATISTICAL TEST USED EPA Probit
 EC50 (MALF.) 1.29 95% CONFIDENCE LIMITS 1.61 - 1.45
 STATISTICAL TEST USED EPA Probit
 TI 1.37

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------|-----------------------|
| NOEL <u>1.5</u> | NOEL <u>1.2</u> | <u>Dunnett's</u> |
| LOEL <u>2</u> | LOEL <u>1.3</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
 MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
 CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)
 COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()
 MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND DMSO CAS # 67-68-5

TEST # 2

CAS #

TEST #

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND DM50 CAS # 67-68-5
 TEST # 3 TEST UNITS V/V%
 FETAX CONTROL MORT. (N,%) 100, 6 FETAX CONT. MALF. (N,%) 94, 6.32
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 CONTROL LENGTH 0.813 cm MCIG 1.2

LC50 (MORT.) 1.86 95% CONFIDENCE LIMITS 1.4 - 2.3
 EC50 (MALF.) 1.24 STATISTICAL TEST USED Litchfield-Wilcoxon
 95% CONFIDENCE LIMITS 0.83 - 1.8
 TI 1.5 STATISTICAL TEST USED Litchfield-Wilcoxon

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------|-----------------------|
| NOEL <u>1.9</u> | NOEL <u>1</u> | <u>Dunnnett</u> |
| LOEL <u>2</u> | LOEL <u>1.2</u> | <u>Dunnnett</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____
 SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____
 MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____
 MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____
 CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)
 COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
 () ()
 MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 EC50 (MALF.) _____ STATISTICAL TEST USED _____
 95% CONFIDENCE LIMITS _____
 TI _____ STATISTICAL TEST USED _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND

DMSO

CAS #

67-68-5

TEST #

3

CONCENTRATION (μ M)

MORTALITY %

MALFORMATION %

Control

6

6.32

.5

8

11.16

.7

6

2.70

.9

12

20.3-

1

12

75

1.2

18

21.9

1.3

18

36.55

1.5

16

76.28

1.7

18

100

1.9

20

100

2.1

4.2

100

2.5

100

~

FETAX SUMMARY SHEET (96hr)

COMPOUND Retinoic Acid CAS # 302-79-4TEST # Definitive 1 TEST UNITS Mg/mlFETAX CONTROL MORT. (N,%) 80, 6.25 FETAX CONT. MALF. (N,%) 75, 4.17

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH .97585 cm MCIG .06

SOL. CONT. LENGTH _____ cm

| | | | |
|--------------|--------------|-----------------------|----------------------------|
| LC50 (MORT.) | <u>.246</u> | 95% CONFIDENCE LIMITS | <u>.216 - .28</u> |
| | | STATISTICAL TEST USED | <u>Litchfield-wilcoxon</u> |
| EC50 (MALF.) | <u>.024</u> | 95% CONFIDENCE LIMITS | <u>.018 - .031</u> |
| | | STATISTICAL TEST USED | <u>Litchfield-wilcoxon</u> |
| TI | <u>10.25</u> | | |

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL <u>.2</u> | NOEL <u>.02</u> | <u>Dunnett's</u> |
| LOEL <u>.3</u> | LOEL <u>.03</u> | <u>Dunnett's</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS + TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

| | | | |
|--------------|-------|-----------------------|-------|
| LC50 (MORT.) | _____ | 95% CONFIDENCE LIMITS | _____ |
| | | STATISTICAL TEST USED | _____ |
| EC50 (MALF.) | _____ | 95% CONFIDENCE LIMITS | _____ |
| | | STATISTICAL TEST USED | _____ |
| TI | _____ | | |

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Retinoic Acid

CAS # 302-79-4

TEST # I

VENDOR/LOT # R-2625/38F-0819 ^{SITING}

CONCENTRATION ($\mu\text{g/ml}$)

MORTALITY %

MALFORMATION %

.005

7.5

2.78

.01

7.5

5.26

102

10

21.78

103

15

53-88

104

7.5

91.13

06

15

100.00

08

1750

100

10

100

15

15

100

30

100

55

100

4

100

工

1

134

FETAX SUMMARY SHEET (96hr)

COMPOUND Retinoic Acid CAS # 302-79-4

TEST # II TEST UNITS µg/ml

FETAX CONTROL MORT. (N,%) 80, 2.5 FETAX CONT. MALF. (N,%) 78, 3.82

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH .944 cm MCIG .08

SOL. CONT. LENGTH _____ cm

| | | | |
|--------------|--------------|-----------------------|----------------------------|
| LC50 (MORT.) | <u>.50</u> | 95% CONFIDENCE LIMITS | <u>.46 - .61</u> |
| | | STATISTICAL TEST USED | <u>EPA Probit</u> |
| EC50 (MALF.) | <u>.044</u> | 95% CONFIDENCE LIMITS | <u>.032 - .0605</u> |
| | | STATISTICAL TEST USED | <u>Litchfield-Wilcoxon</u> |
| TI | <u>11.36</u> | | |

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL <u>.4</u> | NOEL <u>.005</u> | <u>Dunnetts</u> |
| LOEL <u>.5</u> | LOEL <u>.01</u> | <u>Dunnetts</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. (4.0 MG/ML) _____ CYCLOPHOS. CONT. MALF. (4.0 MG/ML) _____

COMAS+TOX. CONT. MORT. () _____ COMAS + TOX. CONT. MALF. () _____

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

| | | | |
|--------------|-------|-----------------------|-------|
| LC50 (MORT.) | _____ | 95% CONFIDENCE LIMITS | _____ |
| | | STATISTICAL TEST USED | _____ |
| EC50 (MALF.) | _____ | 95% CONFIDENCE LIMITS | _____ |
| | | STATISTICAL TEST USED | _____ |
| TI | _____ | | |

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

COMPOUND Retinoic Acid CAS # 302-79-4
TEST # II VENDOR/LOT # ^{Sigma} R-2625/78F-0814

MORTALITY %

MALFORMATION %

.005

7.5

5.41

.01

7.5

18.82

.02

7.5

19.26

०३

7.5

21.76

104

17.5

36.58

.06

25

53.95

03

5-

100.00

-1

12.5

۱۵۰

.15

12.5

100

2

15

100

3

7.5

100

.4

175

100

5.

52.5

100

FETAX SUMMARY SHEET (96hr)

COMPOUND Methyl Mercury Chloride CAS # _____TEST # Range 2 - Definitive 1 TEST UNITS µg/ml = mg/LFETAX CONTROL MORT. (N,%) 80, 6.25 FETAX CONT. MALF. (N,%) 75, 7.24SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —CONTROL LENGTH .97631 cm MCIG 0.036 µg/mlSOL. CONT. LENGTH — cmLC50 (MORT.) 0.0834 95% CONFIDENCE LIMITS 0.08039-0.08671STATISTICAL TEST USED LWEC50 (MALF.) 0.0243 95% CONFIDENCE LIMITS 0.0212 - 0.0279STATISTICAL TEST USED LWTI 3.43MORTALITYNOEL .081LOEL .09MALFORMATIONNOEL .009LOEL .0135STATISTICAL TEST USEDBonferroni T-test" "METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ (4.0 MG/ML) CYCLOPHOS. CONT. MALF. _____ (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ () COMAS + TOX. CONT. MALF. _____ ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

NOEL _____

LOEL _____

MALFORMATION

NOEL _____

LOEL _____

STATISTICAL TEST USED

* Concentration Scaled Based on AA. ANALYSIS

MALFORMATION

| CONC. | <u>9 mo</u> | | TOTAL % | MALE | | TOTAL % |
|---------|-------------|------------|--------------|--------------|--------------|--------------|
| | <u>10</u> | <u>5</u> | | <u>5.56</u> | <u>15.77</u> | |
| CONTROL | <u>0</u> | <u>10</u> | <u>6.25</u> | <u>10</u> | <u>0</u> | <u>7.84</u> |
| 0.009 | <u>10</u> | <u>0</u> | <u>5</u> | <u>11.11</u> | <u>5</u> | <u>8.06</u> |
| 0.0135 | <u>0</u> | <u>0</u> | <u>0</u> | <u>25</u> | <u>30</u> | <u>27.5</u> |
| 0.018 | <u>0</u> | <u>0</u> | <u>0</u> | <u>35</u> | <u>30</u> | <u>32.5</u> |
| 0.027 | <u>0</u> | <u>10</u> | <u>5</u> | <u>35</u> | <u>44.44</u> | <u>39.72</u> |
| 0.036 | <u>5</u> | <u>0</u> | <u>2.5</u> | <u>78.95</u> | <u>60</u> | <u>69.47</u> |
| 0.045 | <u>0</u> | <u>10</u> | <u>5</u> | <u>85</u> | <u>88.89</u> | <u>86.74</u> |
| 0.054 | <u>10</u> | <u>20</u> | <u>15</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.063 | <u>10</u> | <u>5</u> | <u>7.5</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.72 | <u>10</u> | <u>15</u> | <u>12.5</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.081 | <u>0</u> | <u>15</u> | <u>7.5</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.09 | <u>90</u> | <u>80</u> | <u>85.00</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.099 | <u>80</u> | <u>25</u> | <u>77.5</u> | <u>100</u> | <u>100</u> | <u>100</u> |
| 0.18 | <u>100</u> | <u>100</u> | <u>100</u> | <u>100</u> | <u>100</u> | <u>—</u> |
| 0.9 | <u>100</u> | <u>100</u> | <u>100</u> | <u>—</u> | <u>—</u> | <u>—</u> |
| — | — | — | — | — | — | — |
| — | — | — | — | — | — | — |

* Scaled Con content are Actual Based on AB of stock preparation

FETAX SUMMARY SHEET (96hr)

COMPOUND Methyl Mercury chloride CAS # _____

TEST # Def. to 1 (01) TEST UNITS ug/ml or mg/l

FETAX CONTROL MORT. (N,%) 80, 8.75 FETAX CONT. MALF. (N,%) 8.16

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH .91374 cm MCIG .065

SOL. CONT. LENGTH _____ cm

LC50 (MORT.) NA. 105-95 95% CONFIDENCE LIMITS N.P.
 STATISTICAL TEST USED SP2 prob (D. not use official)
 EC50 (MALF.) .021884 95% CONFIDENCE LIMITS .01887 - .025353
 STATISTICAL TEST USED L-W
 TI N2,4

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL <u>.1</u> | NOEL <u>.01</u> | <u>Bonferroni First</u> |
| LOEL <u>4.5</u> | LOEL <u>.015</u> | <u>" "</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
 (4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS + TOX. CONT. MALF. _____
 () ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____
 STATISTICAL TEST USED _____
 TI _____

| <u>MORTALITY</u> | <u>MALFORMATION</u> | <u>STATISTICAL TEST USED</u> |
|------------------|---------------------|------------------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

41 MA-FORMATIONE

| | | 96.1009 | | TOTAL | :MHLF. | | TOTAL % |
|----|----------|---------|-----|-------|--------|-------|---------|
| | | 5 | 0 | | 0 | 15 | |
| 1 | CONTROLE | 15 | 15 | 8.75 | 5.88 | 11.76 | 8.16 |
| 2 | .005 | 0 | 10 | 5 | 10 | 5.50 | 7.78 |
| 3 | .0025 | 5 | 10 | 7.5 | 21.05 | 11.11 | 16.08 |
| 4 | .01 | 15 | 5 | 10 | 17.61 | 21.05 | 19.35 |
| 5 | .015 | 5 | 5 | 5 | 26.72 | 31.58 | 28.95 |
| 6 | .02 | 10 | 15 | 12.50 | 26.32 | 31.58 | 28.95 |
| 7 | .025 | 20 | 10 | 15 | 38.81 | 35.29 | 37.07 |
| 8 | .03 | 0 | 10 | 5 | 75 | 72 | 73.61 |
| 9 | .035 | 15 | 0 | 7.5 | 86 | 66.67 | 73.33 |
| 10 | .04 | 0 | 10 | 50 | 88 | 80 | 84.12 |
| 11 | .045 | 30 | 10 | 20 | 100 | 100 | - |
| 12 | .05 | 5 | 0 | 2.5 | - | - | - |
| 13 | .055 | 10 | 10 | 10 | - | - | - |
| 14 | .06 | 20 | 0 | 10 | - | - | - |
| 15 | .065 | 10 | 20 | 15.0 | - | - | - |
| 16 | .1 | 15 | 0 | 7.5 | - | - | - |
| 17 | .15 | 20 | 100 | 100 | - | - | - |

FETAX SUMMARY SHEET (96hr)

COMPOUND methyl mercury chloride^(P₂) CAS # _____TEST # D2- TEST UNITS µg/mL or mg/LFETAX CONTROL MORT. (N,%) 80, 6.25 FETAX CONT. MALF. (N,%) 9.37SOLVENT CONT. MORT. (N,%) - SOLVENT CONT. MALF. (N,%) -CONTROL LENGTH .95124 cm MCIG .04 mg/L

SOL. CONT. LENGTH _____ cm

LC50 (MORT.) .09409 95% CONFIDENCE LIMITS .0875 - .1011STATISTICAL TEST USED L-WEC50 (MALF.) .02548 95% CONFIDENCE LIMITS .0185 - .03489STATISTICAL TEST USED L-WTI 3.69MORTALITYNOEL .0875LOEL .01MALFORMATIONNOEL .005LOEL .015STATISTICAL TEST USEDBortolow T-Test" "METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ (4.0 MG/ML)
CYCLOPHOS. CONT. MALF. _____ (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ ()
COMAS + TOX. CONT. MALF. _____ ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

NOEL _____

LOEL _____

MALFORMATION

NOEL _____

LOEL _____

STATISTICAL TEST USED

MALFORMATIONS

| CONC. | Q/MO. | | TOTAL | %MALE. | | TOTAL % |
|---------|-------|-----|-------|--------|-------|---------|
| | 5 | 10 | | 5.26 | 11.11 | |
| CONTROL | 10 | 0 | 6.25 | 11.11 | 10 | 9.37 |
| 1005 | 0 | 0 | 0 | 10 | 15 | 12.50 |
| 1015 | 10 | 20 | 15 | 16.67 | 18.75 | 17.71 |
| 102 | 5 | 5 | 5 | 26.32 | 21.08 | 23.68 |
| 1025 | 5 | 15 | 10 | 42.11 | 35.29 | 38.20 |
| 103 | 0 | 20 | 10 | 70 | 68.25 | 69.37 |
| 1035 | 5 | 5 | 5 | 100 | 100 | 100 |
| 104 | 20 | 10 | 15 | - | - | - |
| 105 | 0 | 10 | 5 | - | - | - |
| 1055 | 5 | 0 | 2.5 | - | - | - |
| 1075 | 10 | 5 | 7.5 | - | - | - |
| 108 | 20 | 10 | 15 | - | - | - |
| 10825 | 80 | 0 | 40 | - | - | - |
| 10875 | 10 | 30 | 20 | - | - | - |
| 109 | 10 | 10 | 10 | - | - | - |
| 1 | 100 | 95 | 97.5 | - | - | - |
| 15 | 100 | 100 | 100 | - | - | - |

FETAX SUMMARY SHEET

Test No. Range 1

| | |
|--|---------------------------------|
| Test Material <u>Busulfan</u> | Investigator <u>Lynne Homer</u> |
| Source <u>Aldrich</u> | Lab <u>Bantle</u> |
| CAS No. <u>55-98-1</u> Lot No. <u>6W 0341-1 HV</u> | Test Start Date <u>9-17-90</u> |
| Composition | Test End Date <u>9-21-90</u> |
| Solvent <u>Acetone</u> Conc. <u>1%</u> | Test Units <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 7.0 | 7.0 | 7.0 | — | |
| Control | 7.6 | 7.3 | 7.3 | 7.5 | |
| Highest Conc. | 7.5 | 6.9 | 7.3 | 7.5 | |

| | MORTALITY | MALFORMATION |
|--|--|------------------------------|
| FETAX Control (No./total: %) | <u>7/100</u> : <u>7</u> % | <u>7/93</u> : <u>7.5</u> % |
| Solvent Control | <u>10/50</u> : <u>20</u> % | <u>11/40</u> : <u>27.5</u> % |
| Control Length mm | Solvent Control Length mm | |
| Minimum Concentration to Inhibit Growth (MCIG) | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------------|------------------------|---|------------------------|
| NOEL | | <u>0.10 mg/ml</u> | <u>Booleman T-test</u> |
| LOEL | | <u>0.15 mg/ml</u> | <u>Booleman T-test</u> |
| LC ₅₀ | <u>> 0.20 mg/ml</u> | EC ₅₀ <u>0.16 mg/ml</u> | |
| 95% Confidence limits | | 95% Confidence Limits <u>0.065</u> to <u>0.39 mg/ml</u> | |
| TEST TREATOGENIC INDEX (TI) | | <u>> 1.25</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|----------------------------|----------------------------|
| 5.5 mg/ml | <u>7/50</u> : <u>14</u> % | <u>10/41</u> : <u>22</u> % |
| 2500 mg/ml | <u>8/50</u> : <u>100</u> % | <u>—</u> : <u>—</u> % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | | |
|----------|----------|----------------|----------------|
| Compound | Busulfan | CAS No. | 55-98-1 |
| Test No. | Range 1 | Vendor/Lot No. | BW D3417 HV |

[illegible]

NOT used

FETAX SUMMARY SHEET

Test No. R-3

| | |
|---|---|
| Test Material <i>Furazolidone</i> | Investigator <i>Lynne Homer</i> |
| Source <i>Sigma</i> | Lab <i>Battle</i> |
| CAS No. <i>67-45-8</i> Lot No. <i>47F0405</i> | Test Start Date <i>10/15/90</i> |
| Composition | Test End Date <i>10/19/90</i> |
| Solvent <i>Fetax</i> Conc. <i>0.04 mg/ml</i> | Test Units <i>mg/ml</i> (<i>0.04 mg/ml</i>) |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|------------|------------|------------|------------|------------|
| Stock | <i>6.6</i> | <i>6.6</i> | <i>6.6</i> | <i>6.6</i> | — |
| Control | — | <i>7.2</i> | <i>7.0</i> | | <i>7.6</i> |
| Highest Conc. | — | <i>6.8</i> | <i>6.7</i> | <i>6.8</i> | |

| | MORTALITY | MALFORMATION |
|--|-------------------------------------|---------------------------|
| FETAX Control (No./total: %) | <u>4</u> <u>100</u> : <u>4</u> % | <u>5%</u> : <u>5</u> % |
| Solvent Control | <u>—</u> : <u>—</u> % | <u>—</u> : <u>—</u> % |
| Control Length <u>9.6/60</u> mm | Solvent Control Length <u>—</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.01 mg/ml</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|-----------|--|--------------------------|
| NOEL | | <i>control</i> | <i>Bonferroni T-test</i> |
| LOEL | | <i>0.01 mg/ml</i> | <i>Bonferroni T-test</i> |
| LC ₅₀ <i>~ > 0.018 mg/ml</i> | | EC ₅₀ <i>0.012 mg/ml</i> | |
| 95% Confidence limits | | 95% Confidence Limits <i>1.04 x 10⁻² - 1.47 x 10⁻²</i> | |
| TEST TREATOGENIC INDEX (TI) | | <i>> 1.5</i> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|--------------------------|-----------------------------|
| 5.5 mg/ml | <i>2/2</i> : <i>4</i> % | <i>12/18</i> : <i>25</i> % |
| 2500 mg/ml | <i>3/3</i> : <i>66</i> % | <i>17/17</i> : <i>100</i> % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

GEIHA KAWU DATA SUBVIVIAKI SPCEI (90 PR.)

| | | | |
|----------|---------------------|----------------|----------------|
| Compound | <i>Fluorolidone</i> | CAS No. | <i>67-45-8</i> |
| Test No. | <i>R-3</i> | Vendor/Lot No. | <i>4TF0405</i> |

[illegible]

FETAX SUMMARY SHEET

Test No. Def-1

| | | |
|-----------------------------------|------------------------|---------------------------------|
| Test Material <u>Furazolidone</u> | | Investigator <u>Lynne Homer</u> |
| Source <u>Sigma</u> | | Lab <u>Battle</u> |
| CAS No. <u>67-45-8</u> | Lot No. <u>47F0405</u> | Test Start Date <u>10-22-90</u> |
| Composition | | Test End Date <u>10-26-90</u> |
| Solvent <u>—</u> | Conc. <u>—</u> | Test Units <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 6.5 | 6.5 | 6.5 | 6.5 | |
| Control | | 7.3 | 7.1 | 7.1 | 7.2 |
| Highest Conc. | | 6.9 | 6.3 | 6.5 | |

| | MORTALITY | MALFORMATION |
|---|------------------------------------|-----------------------------|
| FETAX Control (No./total: %) | <u>4/100</u> : <u>4</u> % | <u>19/100</u> : <u>10</u> % |
| Solvent Control | <u>—</u> : <u>—</u> % | <u>—</u> : <u>—</u> % |
| Control Length <u>9.59</u> mm | Solvent Control Length <u>—</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.009 mg/ml</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------------|----------------------|--|--------------------------|
| NOEL | <u>0.012 mg/ml</u> | <u>control</u> | <u>Bonferroni T-test</u> |
| LOEL | <u>0.015 mg/ml</u> | <u>0.009 mg/ml</u> | <u>Bonferroni T-test</u> |
| LC ₅₀ | <u>~ 0.015 mg/ml</u> | EC ₅₀ <u>1.09×10^{-2} mg/ml</u> | |
| 95% Confidence limits | | 95% Confidence Limits <u>$0.010 - 1.182 \times 10^{-2}$ mg/ml</u> | |
| TEST TREATOGENIC INDEX (TI) | | <u>~ 1.38</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|---------------------------|---------------------------|
| 5.5 mg/ml | <u>7/50</u> : <u>4</u> % | <u>1/49</u> : <u>2</u> % |
| 2500 mg/ml | <u>7/50</u> : <u>14</u> % | <u>4/3</u> : <u>100</u> % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | | |
|----------|--------------|----------------|---------|
| Compound | Furazolidone | CAS No. | 67-45-8 |
| Test No. | D-1 | Vendor/Lot No. | 47FO405 |

[illegible]

FETAX SUMMARY SHEET

Test No. D-2

| | |
|---|---------------------------------------|
| Test Material <u>Furazolidone</u> | Investigator <u>James Homer</u> |
| Source <u>Sigma</u> | Lab <u>Bantles</u> |
| CAS No. <u>67-45-8</u> Lot No. <u>47F0405</u> | Test Start Date <u>12-10-90</u> |
| Composition/Purity <u>crystalline</u> | Test End Date <u>12-14-90</u> |
| Solvent _____ Conc. _____ | Test Units (i.e., mg/ml) <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 7.0 | 7.0 | 7.0 | 7.0 | — |
| Control | 6.5 | 7.4 | 7.5 | 7.2 | 7.0 |
| Highest Conc. | — | 7.4 | 7.5 | 7.4 | 7.2 |

| FETAX CONTROL | MORTALITY RECORD | MALFORMATION RECORD |
|---|--|--|
| No. Dead or Malformed Total Number X 100 = % | <u>2/100</u> : <u>.02</u> X 100 = <u>2</u> % | <u>1/100</u> : <u>.01</u> X 100 = <u>1</u> % |
| Solvent Control | <u>—</u> : <u>—</u> X 100 = <u>—</u> % | <u>—</u> : <u>—</u> X 100 = <u>—</u> % |
| Control Length <u>8.99</u> mm | Solvent Control Length <u>—</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.008 mg/ml</u> | | |

TEST MATERIAL/COMPOUND : RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|--------------------|--|--------------------------|
| NOEL | <u>0.012 mg/ml</u> | <u>control</u> | <u>Bonferroni T-test</u> |
| LOEL | <u>0.013 mg/ml</u> | <u>0.009</u> | <u>Bonferroni T-test</u> |
| LC ₅₀ | <u>0.014 mg/ml</u> | EC ₅₀ <u>7.11 x 10⁻³ mg/ml</u> | |
| 95% Confidence limits <u>1.13 x 10⁻² - 1.67 x 10⁻²</u> | | 95% Confidence Limits <u>6.52 x 10⁻³ - 7.76 x 10⁻³</u> | |
| TEST TERATOGENIC INDEX (TI = LC ₅₀ / EC ₅₀) | | | <u>1.97</u> |

POSITIVE CONTROL: 6 AMINONICOTINAMIDE (6-AN) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|---------------|--|---|
| 5.5 mg/L | <u>2/50</u> : <u>.04</u> X 100 = <u>4</u> % | <u>4/50</u> : <u>.08</u> X 100 = <u>8</u> % |
| 2500 mg/L | <u>50/50</u> : <u>1</u> X 100 = <u>100</u> % | <u>—</u> : <u>—</u> X 100 = <u>—</u> % |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | | |
|---------------|---------------------|----------------|----------------|
| Test Material | <i>Funaridolone</i> | CAS No. | <i>67-45-8</i> |
| Test No. | <i>D-2</i> | Vendor/Lot No. | <i>47FC405</i> |

[illegible]

FETAX SUMMARY SHEET

Test No. Def. 1

| | |
|---|---------------------------------|
| Test Material <u>Procarbazine</u> | Investigator <u>Lynne Homer</u> |
| Source <u>Radian</u> | Lab <u>Bartle</u> |
| CAS No. <u>671-16-9</u> Lot No. <u>694686</u> | Test Start Date <u>9-10-90</u> |
| Composition <u>—</u> | Test End Date <u>9-14-90</u> |
| Solvent <u>Fetax</u> Conc. <u>—</u> | Test Units <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 7.7 | 7.7 | 7.7 | 7.8 | — |
| Control | — | 7.7 | 7.3 | 7.0 | 7.3 |
| Highest Conc. | — | 7.7 | 7.8 | 7.4 | 7.7 |

| | MORTALITY | MALFORMATION |
|--|---|---------------------------------|
| FETAX Control (No./total: %) | <u>11/100</u> : <u>11</u> % | <u>4/89</u> : <u>4.5</u> % |
| Solvent Control | <u> </u> : <u> </u> % | <u> </u> : <u> </u> % |
| Control Length <u>9.69</u> mm | Solvent Control Length <u> </u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>1.25 mg/ml</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|---|------------|----------------------------|-----------------------|
| NOEL | 1.75 mg/ml | 1.50 mg/ml | Bartlett's T-test |
| LOEL | 2.00 mg/ml | 1.75 mg/ml | Bartlett's T-test |
| LC ₅₀ | 1.69 mg/ml | EC ₅₀ can't run | |
| 95% Confidence limits 1.420287-2.020287 | | 95% Confidence Limits | |
| TEST TREATOGENIC INDEX (TI) | | | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------------------|----------------------------|
| 5.5 mg/ml | <u>14/50</u> : <u>38</u> % | <u>30/31</u> : <u>97</u> % |
| 2500 mg/ml | <u>50/50</u> : <u>100</u> % | <u>—</u> : <u>—</u> % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | | |
|----------|-------------|----------------|---------------|
| Compound | Procabazine | CAS No. | 671-16-9 |
| Test No. | Def. 1 | Vendor/Lot No. | Radian/694686 |

[illegible]

Test No. D-3

| | |
|---|---------------------------------|
| Test Material <u>Procarrbazine</u> | Investigator <u>Lynne Homer</u> |
| Source <u>Radian</u> | Lab <u>Bantle</u> |
| CAS No. <u>671-16-9</u> Lot No. <u>694686</u> | Test Start Date <u>10-15-90</u> |
| Composition | Test End Date <u>10-19-90</u> |
| Solvent <u>Fetax</u> Conc. <u>4 mg/ml</u> | Test Units <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------|-------|-------|-------|-------|
| Stock | 7.8 | 7.8 | 7.8 | 7.8 | — |
| Control | | 7.2 | 7.0 | 6.9 | 7.3 |
| Highest Conc. | | 7.8 | 7.5 | 7.5 | |

| | MORTALITY | MALFORMATION |
|--|------------------------------------|--------------------------|
| FETAX Control (No./total: %) | <u>4/50</u> : <u>1</u> % | <u>5/49</u> : <u>5</u> % |
| Solvent Control | <u>—</u> : <u>—</u> % | <u>—</u> : <u>—</u> % |
| Control Length <u>9.44</u> mm | Solvent Control Length <u>—</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>1.00 mg/ml</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|--|-------------------|--------------------------|
| NOEL | | <u>control</u> | <u>Bonferroni T-test</u> |
| LOEL | | <u>1.00 mg/ml</u> | <u>Bonferroni T-test</u> |
| LC ₅₀ <u>3.17 mg/ml</u> | EC ₅₀ <u>1.31 mg/ml</u> | | |
| 95% Confidence limits <u>3.10 - 3.24</u> | 95% Confidence Limits <u>1.26 - 1.36</u> | | |
| TEST TREATOGENIC INDEX (TI) | | <u>2.42</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|----------------------------|-----------------------------|
| 5.5 mg/ml | <u>2/50</u> : <u>4</u> % | <u>12/48</u> : <u>25</u> % |
| 2500 mg/ml | <u>33/50</u> : <u>66</u> % | <u>17/17</u> : <u>100</u> % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FEI HA KAW DATA SUMMARIY SHEET (90 Hr.)

| | | | |
|----------|--------------|----------------|----------|
| Compound | Procainazine | CAS No. | 671-16-9 |
| Test No. | D-3 | Vendor/Lot No. | 694686 |

[illegible]

The Raw Data for Trichloroethylene, Acetone and Dimethyl
Sulfoxide Are Contained Within the Text of the Solvent
Interaction Study.

FETAX SUMMARY SHEET (96hr)

R# 1

COMPOUND Trichloroethylene CAS # 79-01-6TEST # 1 TEST UNITS % VclFETAX CONTROL MORT. (N,%) 100 FETAX CONT. MALF. (N,%) 95.2SOLVENT CONT. MORT. (N,%) NA SOLVENT CONT. MALF. (N,%) NA

CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) 0.55 est. 95% CONFIDENCE LIMITS _____EC50 (MALF.) 0.015 est. STATISTICAL TEST USED _____

95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI 3.7 estimatedMORTALITYMALFORMATIONSTATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITYMALFORMATIONSTATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Trichloroethylene CAS # 79-01-6

TEST # Range 1

TEST # Karnal

[illegible]

The Raw Data for 6-Aminonicotinamide is not contained because it was supported by another grant.

Def # 1

FETAX SUMMARY SHEET (96hr)

COMPOUND 113-100-0100 (Ref 3) CAS # 103-90-2TEST # D1 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 10 FETAX CONT. MALF. (N,%) 90, 11.1

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH .837 cm MCIG .1LC50 (MORT.) .146 95% CONFIDENCE LIMITS .122189 - .175435STATISTICAL TEST USED L-WEC50 (MALF.) .1265 95% CONFIDENCE LIMITS .116 - .137STATISTICAL TEST USED L-WTI 1.15

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Acetaminophen - (Act 3) CAS # 103-90-2

ACD minor opior - (Act 3)

103-90-2

DI

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Aluminum (III) Chloride CAS # 107-90-2

TEST # 2 TEST UNITS mg/ml

FETAX CONTROL MORT. (N,%) 100.24 FETAX CONT. MALF. (N,%) 76.17-1

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

CONTROL LENGTH 1.45 cm MCIG 1.12

LC50 (MORT.) .1925 95% CONFIDENCE LIMITS .1609 - .3165

STATISTICAL TEST USED SPSS

EC50 (MALF.) .132 95% CONFIDENCE LIMITS .1196 - .1424

STATISTICAL TEST USED SPSS

TI 1.45

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL N.P.

NOEL N.P.

LOEL N.P.

LOEL N.P.

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)

COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND 1,2-Dichloroethane (100%) CAS # 103-90-2

CAS #

TEST #

MORTALITY %

MALFORMATION %

Cont'd

24

1721

.12

42

42.3

.13

44

57.1

14

35

62.5

22

10

جے

FETAX SUMMARY SHEET (96hr)

COMPOUND Acetaminophen* CAS # 103-90-2TEST # def test DDY TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 25 FETAX CONT. MALF. (N,%) 75, 9SOLVENT CONT. MORT. (N,%) NA SOLVENT CONT. MALF. (N,%) NACONTROL LENGTH .785328 cm MCIG .15LC50 (MORT.) >.30 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) .10 95% CONFIDENCE LIMITS .03-.32STATISTICAL TEST USED Litch/WilcoxTI >3

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL —NOEL .10DunnettsLOEL —LOEL .12Dunnetts**with pen strip*

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL _____

NOEL _____

LOEL _____

LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Acetaminophen w/par CAS # 103-90-2

TEST # dy Test ddy ^{Shrep}

[illegible]

R # 1

FETAX SUMMARY SHEET (96hr)COMPOUND Acetazolamide CAS # 59-66-5TEST # R#1 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 100, 4 FETAX CONT. MALF. (N,%) 96, 3.2SOLVENT CONT. MORT. (N,%) N/A SOLVENT CONT. MALF. (N,%) N/ACONTROL LENGTH N/A cm MCIG N/ASOL. CONT. LENGTH N/A cmLC50 (MORT.) >.1 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) >.1 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI N/AMORTALITYMALFORMATIONSTATISTICAL TEST USEDNOEL DID NOT RUN NOEL _____LOEL DID NOT RUN LOEL _____METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS + TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

MORTALITYMALFORMATIONSTATISTICAL TEST USED

NOEL _____ NOEL _____

LOEL _____ LOEL _____

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Azetazalamide

CAS # 59-66-5

TEST # R # 1

VENDOR/LOT # Sigma / 86F-0075

CONCENTRATION (mg/ml)

MORTALITY %

MALFORMATION %

Control

4

3.2

0001

4.

8.3

10065

4

12.5

0010

6

12.8

: 0050

3

15.4

10100

2

14.4

0550

4

16.7

. 1500

6

19.6

FETAX SUMMARY SHEET (96hr)

COMPOUND Benzo (a) Pyrene CAS # 50-38-2TEST # 1 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 0^{DMSO}
SOLVENT CONT. MORT. (N,%) 40, 2.5 SOLVENT CONT. MALF. (N,%) 39, 5%CONTROL LENGTH 0.8600 cm MCIG —LC50 (MORT.) >0.01 95% CONFIDENCE LIMITS —STATISTICAL TEST USED —EC50 (MALF.) 0.01 95% CONFIDENCE LIMITS (.008 - .011)STATISTICAL TEST USED Litchfield/WilcoxonTI >1

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL >.01NOEL .0050Dunnett'sLOEL >.01LOEL .0075Dunnett's

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 0%^{DMSO}
SOLVENT CONT. MORT. (N,%) 40, 2.5 SOLVENT CONT. MALF. (N,%) 39, 5%MAS CONT. MORT. (N,%) 40, 0 MAS CONT. MALF. (N,%) 40, 0%MAS+SOL. CONT. MORT. (N,%) 40, 0 MAS+SOL. CONT. MALF. (N,%) 40, 15%CYCLOPHOS. CONT. MORT. 40, 100 CYCLOPHOS. CONT. MALF. — —
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. 40, 0 COMAS+TOX. CONT. MALF. 40, 40%
(10 ug/ml) (10 ug/ml)MAS+SOLVENT CONTROL LENGTH .8500 cm MCIG —LC50 (MORT.) >0.01 95% CONFIDENCE LIMITS —STATISTICAL TEST USED —EC50 (MALF.) 0.002 95% CONFIDENCE LIMITS (.001 - 0.0024)STATISTICAL TEST USED Litchfield/WilcoxonTI >5.0

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL .001NOEL >0.01Dunnett'sLOEL .005LOEL >0.01Dunnett's

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Benzo (a) Pyrene CAS # 50-38-2

TEST # 1

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Benzo(a)Pyrene CAS # 50-38-2TEST # 2 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 3.8SOLVENT CONT. MORT. (N,%) 40, 2.5 SOLVENT CONT. MALF. (N,%) 39, 5.1CONTROL LENGTH .8500 cm MCIG —

LC50 (MORT.) > .01 95% CONFIDENCE LIMITS —
 EC50 (MALF.) 0.012 STATISTICAL TEST USED —
 95% CONFIDENCE LIMITS (.006 - .02)
 TI < 1.0 STATISTICAL TEST USED Litchfield/Wilcoxon

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|----------------------|------------------|-----------------------|
| NOEL <u>> .01</u> | NOEL <u>.001</u> | <u>Dunnetts</u> |
| LOEL <u>> 0.1</u> | LOEL <u>.005</u> | <u>Dunnetts</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 80, 0 FETAX CONT. MALF. (N,%) 80, 3.8SOLVENT CONT. MORT. (N,%) 40, 2.5 SOLVENT CONT. MALF. (N,%) 39, 5.1MAS CONT. MORT. (N,%) 40, 2.5 MAS CONT. MALF. (N,%) 39, 2.6MAS+SOL. CONT. MORT. (N,%) 40, 0 MAS+SOL. CONT. MALF. (N,%) 40, 5CYCLOPHOS. CONT. MORT. 40, 100 CYCLOPHOS. CONT. MALF. — —
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. 40, 0 COMAS+TOX. CONT. MALF. 40, 25%
(10 ug/ml) (10 ug/ml)MAS+SOLVENT CONTROL LENGTH .775 cm MCIG —

LC50 (MORT.) > 0.01 95% CONFIDENCE LIMITS —
 EC50 (MALF.) 0.0018 STATISTICAL TEST USED —
 95% CONFIDENCE LIMITS (.0012 - .003)
 TI > 5.6 STATISTICAL TEST USED Litchfield/Wilcoxon

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|---------------------|------------------------|-----------------------|
| NOEL <u>0.01</u> | NOEL <u>< .0005</u> | <u>Dunnetts</u> |
| LOEL <u>> 01</u> | LOEL <u>.0005</u> | <u>Dunnetts</u> |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Benzo (α) Pyrone

CAS # 50-38-Z

TEST # 2

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Dimethyl N-methylamine CAS # 62-75-9TEST # Def1 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 80, 1.2% FETAX CONT. MALF. (N,%) 79, 1.3%SOLVENT CONT. MORT. (N,%) NA SOLVENT CONT. MALF. (N,%) NACONTROL LENGTH 0.93065 cm MCIG 2.15LC50 (MORT.) 3.53 95% CONFIDENCE LIMITS 3.33 - 3.65EC50 (MALF.) 2.26 STATISTICAL TEST USED EPA PROBT95% CONFIDENCE LIMITS 2.15 - 2.38STATISTICAL TEST USED Likelihood WilcoxonTI 1.66

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------------|-----------------------|
| NOEL <u>3.0</u> | NOEL <u>< 1.75</u> | <u>Dunnetts</u> |
| LOEL <u>3.5</u> | LOEL <u>1.75</u> | <u>Dunnetts</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) _____ FETAX CONT. MALF. (N,%) _____

SOLVENT CONT. MORT. (N,%) _____ SOLVENT CONT. MALF. (N,%) _____

MAS CONT. MORT. (N,%) _____ MAS CONT. MALF. (N,%) _____

MAS+SOL. CONT. MORT. (N,%) _____ MAS+SOL. CONT. MALF. (N,%) _____

CYCLOPHOS. CONT. MORT. _____ CYCLOPHOS. CONT. MALF. _____
(4.0 MG/ML) (4.0 MG/ML)COMAS+TOX. CONT. MORT. _____ COMAS+TOX. CONT. MALF. _____
() ()

MAS+SOLVENT CONTROL LENGTH _____ cm MCIG _____

LC50 (MORT.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

EC50 (MALF.) _____ 95% CONFIDENCE LIMITS _____

STATISTICAL TEST USED _____

TI _____

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|------------|--------------|-----------------------|
| NOEL _____ | NOEL _____ | _____ |
| LOEL _____ | LOEL _____ | _____ |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Dimethyl Nitrosamine

CAS # 62-75-9

TEST # Test 1

CONCENTRATION (mg/ml)

MORTALITY %

MALFORMATION %

(Control)

1.3 %

1.3 %

1.5

0

10%

1.75

0

17.5 %

2.0

0

20 %

2.25

0

42.5 %

2.5

0

60. %

2.75

0

80. %

3.0

0

80 %

3.5

48 %

100 %

4.0

95 %

100 %

4.25

90 %

100 %

4.5

100 %

NH

4.75

100 %

5.0

100 %

5.25

100 %

5.5

100 %

5.75

100 %

6.0

100 %

6.5

100 %

7.0

100 %

✓

FETAX SUMMARY SHEET (96hr)

COMPOUND Sodium Salicylate CAS # 54-21-7TEST # 1 TEST UNITS mg/mlFETAX CONTROL MORT. (N,%) 80, 5 FETAX CONT. MALF. (N,%) 76, 8.8SOLVENT CONT. MORT. (N,%) - SOLVENT CONT. MALF. (N,%) -CONTROL LENGTH 0.89 cm MCIG 1.25LC50 (MORT.) 2.34 95% CONFIDENCE LIMITS 1.92 - 2.86EC50 (MALF.) 1.67 STATISTICAL TEST USED L/W95% CONFIDENCE LIMITS 1.32 - 2.11STATISTICAL TEST USED L/WTI 1.4

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL 1.25NOEL 1.0DUNN & TTSLOEL 2.25LOEL 1.25"

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 80, 5 ^{1.25} FETAX CONT. MALF. (N,%) 79, 6.3SOLVENT CONT. MORT. (N,%) - SOLVENT CONT. MALF. (N,%) -MAS CONT. MORT. (N,%) 80, 2.5 MAS CONT. MALF. (N,%) 78, 5.3MAS+SOL. CONT. MORT. (N,%) - MAS+SOL. CONT. MALF. (N,%) -CYCLOPHOS. CONT. MORT. 40, 100 (4.0 MG/ML) CYCLOPHOS. CONT. MALF. 0, - (4.0 MG/ML)COMAS+TOX. CONT. MORT. 47.5 (2.0) COMAS+TOX. CONT. MALF. 100 (2.0)MAS+SOLVENT CONTROL LENGTH 0.85 cm MCIG 1.2LC50 (MORT.) 2.19 95% CONFIDENCE LIMITS 2.04 - 2.34STATISTICAL TEST USED L/WEC50 (MALF.) 1.37 95% CONFIDENCE LIMITS 1.30 - 1.44STATISTICAL TEST USED L/WTI 1.6

MORTALITY

MALFORMATION

STATISTICAL TEST USED

NOEL 1.4NOEL 1.4DUNN & TTSLOEL 1.6LOEL 1.6"

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Sodium Salicylate CAS # 54-97-5

TEST # DEF I - MAS

[illegible]

FETAX SUMMARY SHEET (96hr)

COMPOUND Sodium Salicylate CAS # 54-21-7
 TEST # DGF 2 TEST UNITS mg/ml
 FETAX CONTROL MORT. (N,%) 80, 2.5 FETAX CONT. MALF. (N,%) 78, 2.6
 SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —
 CONTROL LENGTH 0.88 cm MCIG 1.0
 LC50 (MORT.) 2.30 95% CONFIDENCE LIMITS 2.23-2.36
 EC50 (MALF.) 1.23 STATISTICAL TEST USED H/W
 95% CONFIDENCE LIMITS 1.16-1.3
 STATISTICAL TEST USED H/W
 TI 1.9

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------|-----------------------|
| NOEL <u>2.0</u> | NOEL <u>1.2</u> | <u>DUNNETT'S</u> |
| LOEL <u>2.1</u> | LOEL <u>1.4</u> | <u>"</u> |

METABOLIC ACTIVATION

FETAX CONT. MORT. (N,%) 80, 5 FETAX CONT. MALF. (N,%) 76, 0
 SOLVENT CONT. MORT. (N,%) — SOLVENT CONT. MALF. (N,%) —
 MAS CONT. MORT. (N,%) 40, 0 MAS CONT. MALF. (N,%) 40, 0
 MAS+SOL. CONT. MORT. (N,%) — MAS+SOL. CONT. MALF. (N,%) —
 CYCLOPHOS. CONT. MORT. 40, 100 CYCLOPHOS. CONT. MALF. 40, 00
 (4.0 MG/ML) (4.0 MG/ML)
 COMAS+TOX. CONT. MORT. 35 COMAS+TOX. CONT. MALF. 94
 (2.0) (2.0)
 MAS+SOLVENT CONTROL LENGTH 0.87 cm MCIG 1.2
 LC50 (MORT.) 2.25 95% CONFIDENCE LIMITS 2.16-2.35
 EC50 (MALF.) 1.49 STATISTICAL TEST USED H/W
 95% CONFIDENCE LIMITS 1.42-1.57
 STATISTICAL TEST USED H/W
 TI 1.5

| MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------|-----------------|-----------------------|
| NOEL <u>1.4</u> | NOEL <u>1.2</u> | <u>DUNNETT'S</u> |
| LOEL <u>1.6</u> | LOEL <u>1.4</u> | <u>"</u> |

RAW DATA SUMMARY SHEET (96hr)

COMPOUND Sodium Salicylate CAS #

TEST # DGP 2 - MAS

| CONCENTRATION (mg/ml) | MORTALITY % | MALFORMATION % |
|-----------------------|-------------|----------------|
| FGTAX SOLIN | 0 | 5 |
| MAS | 0 | 0 |
| CO-MAS + 2.0 | 35 | 93.8 |
| 4.0 mg/ml CP | 100 | — |
| 1.2 | 7.5 | 19.7 |
| 1.4 | 10 | 38.7 |
| 1.6 | 10 | 50.2 |
| 1.8 | 17.5 | 72.8 |
| 2 | 32.5 | 96.2 |
| 2.1 | 37.5 | 100 |
| 2.2 | 45 | 100 |
| 2.3 | 47.5 | 100 |
| 2.4 | 55 | 100 |
| 2.5 | 72.5 | 100 |

FETAX SUMMARY SHEET

Test No. D-16

| | |
|-------------------------------------|--------------------------------|
| Test Material <u>Sodium Acetate</u> | Investigator <u>DeYoung</u> |
| Source | Lab <u>Battle</u> |
| CAS No. | Lot No. |
| Composition | Test Start Date <u>7/16/90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> |
| | Test End Date <u>7/21/90</u> |
| | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|----------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>7.4-8.1</u> | | | | |
| Control | <u>L</u> | | | | |
| Highest Conc. | <u>L</u> | | | | |

| | MORTALITY | MALFORMATION |
|---|--|----------------------------|
| FETAX Control (No./total: %) | <u>8/80</u> : <u>10</u> % | <u>1/72</u> : <u>3.1</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>1.01185</u> | Solvent Control Length mm <u>NA</u> | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>2.5</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------------|------------------|-----------------------|---|
| NOEL | <u>3.0</u> | <u>2.5</u> | <u>Anova-Bonferroni</u> |
| LOEL | <u>3.25</u> | <u>3.0</u> | " " |
| LC ₅₀ | <u>4.30</u> | EC ₅₀ | <u>3.26</u> |
| 95% Confidence limits | <u>3.80-4.87</u> | 95% Confidence Limits | <u>3.00-3.56</u> |
| TEST TREATOGENIC INDEX (TI) | | <u>1.9</u> | <u>4dyLC50 = 4.33</u> <u>3.82-4.90</u> |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | |
|---------------|----------------------|----------------|
| Test Material | Sodium Acetate | CAS No. |
| Test No. | Def. 1 - Xenopus Sdy | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-2

| | |
|-------------------------------------|--------------------------------|
| Test Material <u>Sodium acetate</u> | Investigator <u>Delfeung</u> |
| Source | Lab <u>Bantle</u> |
| CAS No. | Lot No. |
| Composition | Test Start Date <u>8/12/90</u> |
| Solvent <u>NA</u> | Test End Date <u>8/17/90</u> |
| Conc. <u>NA</u> | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-----------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | 7.4-8.1 → | | | | |
| Control | ↳ | | | | |
| Highest Conc. | ↳ | | | | |

| | MORTALITY | MALFORMATION |
|---|-------------------------------------|----------------------------|
| FETAX Control (No./total: %) | <u>2/80</u> : <u>2.5</u> % | <u>4/78</u> : <u>5.1</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>1.00696</u> | Solvent Control Length <u>NA</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>2.5</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|------------------------------|--|---------------------------------|
| NOEL | <u>4.25</u> | <u>3.0</u> | <u>anova - bonferroni</u> |
| LOEL | <u>4.5</u> | <u>3.75</u> | <u>"</u> |
| LC ₅₀ <u>4.34</u> | EC ₅₀ <u>3.33</u> | | |
| 95% Confidence limits <u>(3.57-5.27)</u> | | 95% Confidence Limits <u>(2.76-4.01)</u> | |
| TEST TREATOGENIC INDEX (TI) <u>0.77</u> | | <u>1.8</u> | <u>4du - CS3</u> <u>4.06</u> |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | |
|---------------|-------------------|----------------|
| Test Material | Sodium Acetate | CAS No. |
| Test No. | def 2 Xenopus Sdy | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. 0-1

| | |
|-------------------------------------|--------------------------------|
| Test Material <u>Sodium Acetate</u> | Investigator <u>Delfour</u> |
| Source | Lab <u>Bantle</u> |
| CAS No. | Lot No. |
| Composition | Test Start Date <u>8-28-90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> |
| | Test End Date <u>8-33-90</u> |
| | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>~8.2</u> | | | | |
| Control | <u>↓</u> | | | | |
| Highest Conc. | | | | | |

| | MORTALITY | MALFORMATION |
|--|-------------------------------------|----------------------------|
| FETAX Control (No./total: %) | <u>1/90</u> : <u>1.3</u> % | <u>1/79</u> : <u>1.3</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>2.48089</u> | Solvent Control Length <u>NA</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>< 7.0</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|---|--------------|---------------------------|
| NOEL | <u>13.0</u> | <u>8.0</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>15.0</u> | <u>9.0</u> | <u>11</u> |
| LC ₅₀ <u>13.53</u> | EC ₅₀ <u>9.21</u> | | |
| 95% Confidence limits <u>11.29 - 16.21</u> | 95% Confidence Limits <u>7.24 - 10.67</u> | | |
| TEST TREATOGENIC INDEX (TI) | | <u>1.5</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | |
|---------------|--------------------|----------------|
| Test Material | Sodium Acetate | CAS No. |
| Test No. | Def 1 - Pimephales | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-1 FROG

| | | |
|-------------------------------|-----------------|--------------------------------|
| Test Material <u>Caffeine</u> | | Investigator <u>Delfourng</u> |
| Source | | Lab <u>Bartle</u> |
| CAS No. | Lot No. | Test Start Date <u>6/13/90</u> |
| Composition | | Test End Date <u>6/18/90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|----------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>7.5-7.8</u> | | | | |
| Control | <u>L</u> | | | | |
| Highest Conc. | <u>L</u> | | | | |

| | MORTALITY | MALFORMATION |
|---|---|----------------------------|
| FETAX Control (No./total: %) | <u>3/80</u> : <u>3.8</u> % | <u>4/77</u> : <u>5.2</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>1.00369</u> | Solvent Control Length mm <u>NA</u> | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.1</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|--|--------------|---|
| NOEL | <u>0.18</u> | <u>0.07</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>0.20</u> | <u>0.10</u> | <u>" "</u> |
| LC ₅₀ <u>0.18</u> | EC ₅₀ <u>0.12</u> | | |
| 95% Confidence limits <u>0.18-0.19</u> | 95% Confidence Limits <u>0.11-0.13</u> | | |
| TEST TREATOGENIC INDEX (TI) | | <u>1.5</u> | <u>4dyLC50 0.20</u> <u>0.16-0.25</u> |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | |
|---------------|---------------------|----------------|
| Test Material | Caffeine | CAS No. |
| Test No. | Def 1 - Xenopus Sdy | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-2 FROE

| | | | |
|---------------|-----------------|-----------------|----------------|
| Test Material | <u>Caffeine</u> | Investigator | <u>DeYoung</u> |
| Source | | Lab | <u>Bantle</u> |
| CAS No. | Lot No. | Test Start Date | <u>6/13/90</u> |
| Composition | | Test End Date | <u>6/18/90</u> |
| Solvent | <u>NA</u> | Conc. | <u>NA</u> |
| | | Test Units | <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-----------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | 7.6 → | | | | |
| Control | 7.5-7.7 → | | | | |
| Highest Conc. | 7.8 → | | | | |

| | MORTALITY | MALFORMATION |
|--|--|----------------------------|
| FETAX Control (No./total: %) | <u>5/80</u> : <u>6.3</u> % | <u>1/75</u> : <u>1.3</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>0.99261</u> | Solvent Control Length mm <u>NA</u> | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.05</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------------|-------------------------------|-----------------------|---------------------------|
| NOEL | <u>> .2</u> ^{1.0} | <u>.07</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>> .2</u> ^{1.0} | <u>.10</u> | <u>" "</u> |
| LC ₅₀ | <u>0.22</u> | EC ₅₀ | <u>0.13</u> |
| 95% Confidence limits | <u>0.21 - 0.23</u> | 95% Confidence Limits | <u>0.12 - 0.15</u> |
| TEST TREATOGENIC INDEX (TI) | | <u>1.7</u> | |

CONTROL: 6 AMINGNICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | | |
|---------------|-------------------|----------------|
| Test Material | Caffeine | CAS No. |
| Test No. | Def 2 Xenopus Sdv | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-1 FISH

| | | |
|-------------------------------|-----------------|--------------------------------|
| Test Material <u>Caffeine</u> | | Investigator <u>Delfung</u> |
| Source | | Lab <u>Bantle</u> |
| CAS No. | Lot No. | Test Start Date <u>8/21/90</u> |
| Composition | | Test End Date <u>8/26/90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>~7.5</u> | | | | |
| Control | <u>F</u> | | | | |
| Highest Conc. | | | | | |

| | MORTALITY | MALFORMATION |
|--|-------------------------------------|----------------------------|
| FETAX Control (No./total: %) | <u>4/80</u> : <u>5</u> % | <u>1/76</u> : <u>1.3</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm , <u>47638</u> | Solvent Control Length <u>NA</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.02</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|-----------------------------|-------------------------------------|-----------------------|---------------------------|
| NOEL | <u>>7</u> | <u>0.02</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>>7</u> ^{not reached} | <u>0.04</u> | <u>Anova - Bonferroni</u> |
| LC ₅₀ | <u>0.76</u> | EC ₅₀ | <u>0.10</u> |
| 95% Confidence limits | <u>0.59 - 2.3⁻⁹</u> | 95% Confidence Limits | <u>0.08 - 0.13</u> |
| TEST TREATOGENIC INDEX (TI) | | <u>12.9</u> | <u>4 day LC50 = 4.93</u> |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | |
|-------------------------------------|----------------|
| Test Material <i>Caffeine</i> | CAS No. |
| Test No. <i>Def 1 - pine phates</i> | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-1 FROG

| | |
|-------------------------------------|--------------------------------|
| Test Material <u>5-Fluorouracil</u> | Investigator <u>DeYoung</u> |
| Source | Lab <u>Bantle</u> |
| CAS No. | Lot No. |
| Composition | Test Start Date <u>6/13/90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> |
| | Test End Date <u>6/18/90</u> |
| | Test Units <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>~7.7</u> | | | | |
| Control | <u>~8.1</u> | | | | |
| Highest Conc. | <u>7.5</u> | | | | |

| | MORTALITY | MALFORMATION |
|--|-------------------------------------|---------------------|
| FETAX Control (No./total: %) | <u>7/80 : 8.8 %</u> | <u>1/73 : 1.4 %</u> |
| Solvent Control | <u>NA : NA %</u> | <u>NA : NA %</u> |
| Control Length mm <u>1.05193</u> | Solvent Control Length <u>NA</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>0.25</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|--|--|-------------------------|
| NOEL | <u>0.25</u> | <u>0.05</u> | <u>Anova Bonferroni</u> |
| LOEL | <u>0.30</u> | <u>0.06</u> | <u>"</u> |
| LC ₅₀ <u>0.48</u> | EC ₅₀ <u>0.088</u> | | |
| 95% Confidence limits <u>0.26-0.89</u> | 95% Confidence Limits <u>0.077-0.010</u> | | |
| TEST TREATOGENIC INDEX (TI) | <u>5.5</u> | <u>7dy LC50 1.02</u> <u>0.91-1.14</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | |
|-------------------------------------|----------------|
| Test Material <i>5-Fluorouracil</i> | CAS No. |
| Test No. <i>Def 1 Xenopus 5dy</i> | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-2 Frog

| | | | |
|---------------|-----------------------|-----------------|----------------|
| Test Material | <u>5-Fluorouracil</u> | Investigator | <u>DeJours</u> |
| Source | | Lab | <u>Bancro</u> |
| CAS No. | Lot No. | Test Start Date | <u>7/16/90</u> |
| Composition | | Test End Date | <u>7/21/90</u> |
| Solvent | <u>NA</u> | Conc. | <u>NA</u> |
| | | Test Units | <u>mg/ml</u> |

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|-------------|-------|-------|-------|-------|
| — pH — | | | | | |
| Stock | <u>~7.5</u> | | | | |
| Control | <u>1</u> | | | | |
| Highest Conc. | | | | | |

| | MORTALITY | MALFORMATION |
|---|-------------------------------------|--------------------------|
| FETAX Control (No./total: %) | <u>18/80</u> : <u>22.5</u> % | <u>0/62</u> : <u>0</u> % |
| Solvent Control | <u>NA</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>0.98017</u> | Solvent Control Length <u>NA</u> mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u><.05</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|-------------|---|--|
| NOEL | <u>0.5</u> | <u><.05</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>1.0</u> | <u>.05</u> | <u>"</u> |
| LC ₅₀ | <u>0.62</u> | EC ₅₀ | <u>0.055</u> |
| 95% Confidence limits <u>0.49 - 0.71</u> | | 95% Confidence Limits <u>0.49 - 0.611</u> | |
| TEST TREATOGENIC INDEX (TI) | | <u>11.3</u> | <u>4 day LC50 0.73</u> <u>0.61 - 0.59</u> |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |

FETAX RAW DATA SUMMARY SHEET (96 Hr.)

| | |
|-------------------------------------|----------------|
| Test Material <i>5-fluorouracil</i> | CAS No. |
| Test No. <i>Def 2 - Xenopus</i> | Vendor/Lot No. |

[illegible]

FETAX SUMMARY SHEET

Test No. D-1 fresh

| | |
|-------------------------------------|--------------------------------|
| Test Material <u>5-fluorouracil</u> | Investigator <u>DeYoung</u> |
| Source | Lab <u>Battle</u> |
| CAS No. | Lot No. |
| Composition | Test Start Date <u>8/30/90</u> |
| Solvent <u>NA</u> | Conc. <u>NA</u> |
| | Test End Date <u>9/3/90</u> |
| | Test Units <u>mg/ml</u> |

| — pH — | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---------------|--------------|-------|-------|-------|-------|
| Stock | <u>7.5-8</u> | | | | |
| Control | <u>—</u> | | | | |
| Highest Conc. | | | | | |

| | MORTALITY | MALFORMATION |
|---|----------------------------|----------------------------|
| FETAX Control (No./total: %) | <u>2/40</u> : <u>2.5</u> % | <u>1/78</u> : <u>1.3</u> % |
| Solvent Control | <u>1/1</u> : <u>NA</u> % | <u>NA</u> : <u>NA</u> % |
| Control Length mm <u>0.45185</u> | Solvent Control Length mm | |
| Minimum Concentration to Inhibit Growth (MCIG) <u>< .1</u> | | |

TEST MATERIAL/COMPOUND RESULTS

| TEST | MORTALITY | MALFORMATION | STATISTICAL TEST USED |
|--|--|-----------------|---------------------------|
| NOEL | <u>0.3</u> | <u>< 0.1</u> | <u>Anova - Bonferroni</u> |
| LOEL | <u>0.5</u> | <u>< 0.1</u> | <u>"</u> |
| LC ₅₀ <u>1.33</u> | EC ₅₀ <u>0.15</u> | | |
| 95% Confidence limits <u>1.12 - 1.58</u> | 95% Confidence Limits <u>0.11 - 0.21</u> | | |
| TEST TREATOGENIC INDEX (TI) | | <u>6.9</u> | |

CONTROL: 6 AMINONICOTINAMIDE (ANT) RESULTS

| CONCENTRATION | MORTALITY | MALFORMATION |
|--------------------------------------|-----------------|-----------------|
| 5.5 mg/ml | _____ : _____ % | _____ : _____ % |
| 2500 mg/ml | _____ : _____ % | _____ : _____ % |
| CONTROL (ANT) TERATOGENIC INDEX (TI) | | |